IMPORTANT NUCLEOTIDE SEQUENCES INVOLVED IN LATENCY OF DNA VIRUSES OF ANIMALS

By

Mark A. Rayfield

A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL
OF THE UNIVERSITY OF FLORIDA
PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1982

This work is dedicated to my family: to my parents Martin and Fran whose sacrifices made it all possible, to Glenn who shared the lean years and to my wife Dot for her loving support of a very tired and grouchy graduate student.....

ACKNOWLEDGEMENTS

I would like to acknowledge the efforts of Tess Korhnak and Dr. Vishwanath Suryanarayanan with whom I've had the good fortune to be associated during these studies. I would also like to take this opportunity to express my appreciation to the members of my advisory committee, especially Dr. Kenneth I. Berns. A warm thanks is extended to Drs. Bill Hauswirth, Bill Hollowman and Phil Laipis who have continually supplied good natured advice and whose laboratories have been a warehouse of materials and equipment. Finally, I would like to thank my wife Dot and Gail Crummer, a masochist but dear friend, for their selfless contributions to the organization and drafting of this manuscript.

TABLE OF CONTENTS

SECTION	PAGE
ACKNOWLEDGEMENTS	iv
ABSTRACT	vii
INTRODUCTION	1
Molecular Biology of HSV	7
Genome Size and Composition	7
Nucleotide Sequence Arrangement	10
Endonuclease Cleavage Map	13
Transcription	14
MATERIALS AND METHODS	20
Purification of Ganglion Cell DNA and RNA	20
Viral DNA Purification	20
Preparation of Cloned DNA	22
DNA Restriction	26
Gel Electrophoresis	26
Isolation of Endonuclease Cleavage Products	27
Mapping Approach	28
Nick Translation	30
Iodination of Cytoplasmic RNA	31
5' End Labeling Reactions	31
Southern Blots	32
Hybridization Reaction	33
RESULTS	35
Experimental Approach	35
Controls	37
Hybridization of Ganglia Nucleic Acids to HSV DNA	41
CsCl Enrichment Studies	44
Mapping the L/S junction of HSV-1 F strain	49
HYBRIDIZATIONS	59
Hybridization of ganglia RNA to the L/S junction	59
Cross Hybridization Experiments	60
DISCUSSION	75

LIST OF REFERENCES 88
BIOGRAPHICAL SKETCH 98

Abstract of Dissertation Presented to the Graduate Council of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

IMPORTANT NUCLEOTIDE SEQUENCES INVOLVED IN LATENCY OF DNA VIRUSES OF ANIMALS

By

Mark A. Rayfield

December 1982

Chairman: Kenneth I. Berns, Ph.D., M.D.
Major Department: Immunology and Medical Microbiology

We have investigated the molecular status of the Herpes simplex virus (HSV) genome in latently infected human trigeminal ganglia. The general aim of this research is to illuminate the genetics of viral latency during recurrent infections in man. The trigeminal ganglia of 22 patients have been assayed for HSV sequences by DNA/DNA or RNA/DNA hybridization. None of the patients manifested clinical evidence of recurrence at the time of death nor was a history of recurrent HSV lesions noted. Fourteen of the patients were sero-positive for HSV. Positive serology was assessed as a complement fixation titer of 1:8 or greater. In 18 of the 22 assays in which trigeminal ganglia were evaluated for the presence of HSV, total ganglionic DNA was cleaved by endonuclease digestion, transferred to nitrocellulose and probed with rich nick-translated HSV DNA. The nucleic acid from two of the sero-positive

patients failed to demonstrate HSV specific sequences in either DNA or RNA. Only one of the eight sero-negative patients gave evidence of the presence of HSV DNA or RNA, the remainder were negative. Thus, there is a positive correlation between the presence of complement fixing antibodies and the presence of sequestered HSV specific sequences as measured by these techniques. Further, from the mapping of those RNA transcripts observed, it is clear that the regions of the genome most frequently encountered to date are those containing sequences in the immediate early and early mRNA species of HSV-1.

INTRODUCTION

The involvement of herpes simplex virus (HSV) in recurrent infection of man is well documented. These recurrences are most frequently manifested as periodic ulcerations of the oral or genital mucosa and infections of adjacent cutaneous tissues. Recurrent herpetic keratitis is, today, the most common severe ocular infection in the United States and is the principle factor involved in blinding corneal disease. Ocular infections in 50% of the patients experiencing a primary episode will recur within 2 years. Latent virus is the probable source of infection producing acute episodes of HSV encephalitis and meningitis in adults. It also serves as a reservoir for virus involved in generalized herpetic infections in newborns and is potentially involved in carcinoma of the cervix.

Several lines of evidence suggest the involvement of neural tissue with viral latency in man. Repetitive infections generally occur in localized tissues which define together the limits of a region innervated by a single sensory ganglia. Trauma to the sensory nerve will consistently induce a viral recurrence in peripheral tissue. Reactivation occurs in greater than 90% of the patients undergoing root sections of the trigeminal nerve; none is observed after trauma to tissue previously denervated (Carton and Kilborne, 1952). A majority of the patients who reactivate following neurosurgical treatment for trigeminal neuralgia have a history of recurrent herpes labialis (Pazin et al., 1978). While such observations are only indirect evidence that neural tissue is the site of latency, it is noteworthy that the significant efforts by

investigators to demonstrate virus in peripheral tissues during quiescent periods have failed (Rustigan et al., 1966; Baringer, 1975).

HSV can be liberated from cultures of latently infected trigeminal ganglia from unselected humans (Bastian et al., 1972; Baringer, 1974). Using tissue cocultivation with HSV susceptible cell lines or organotypic cultures for periods of 10-45 days in vitro, the vagus, sacral and superior cervical ganglia may also be shown to harbor virus in some individuals (Baringer 1974, 1975; Warren et al., 1978; Lonsdale et al., 1979). EM studies show virions and cytopathology similar to that of HSV in such cultures. The virus isolated may be identified as HSV on the basis of both serologic (Bastian et al., 1972; Baringer, 1975) and genetic criteria (Lonsdale et al., 1979). Similar culture techniques fail to yield virus from the choroid plexus, trigeminal nerve root or cutaneous tissues from known positive individuals. This argues that the ganglion may be the sole site of latency.

Several parameters of latency can be envisioned from studies of the virus yielded from explants. Serological evaluations of isolates suggest that the virus remains phenotypically unaltered during latency; reactivated virus may at some point spread to adjacent ganglia and become latent in these; ganglia innervating remote peripheral sites may infrequently become latent with seriologically distinct strains of the virus; finally, both HSV type I and II may be latent and recur within the same individual (Stevens and Cook, 1971; Baringer, 1975). Analysis of the restriction endonuclease digestion patterns of their DNA and gel electrophoresis patterns of isolates proteins confirms these observations (Lonsdale et al., 1979; Buchman et al., 1978). Further, such studies have shown that the isolates were unique to an individual unless they

were epidemiologically related and that within the individual multiple isolates from different ganglia were usually genetically identical. Infection of peripheral ganglia appears to be subsequent to viral spread from the site of initial infection in a majority of individuals. These observations suggest that once a sensory ganglion cell becomes latently infected it is to some degree refractory to superinfection by a future viral isolate. Complementation studies involving superinfection of ganglia with temperature sensitive mutants of HSV indicate that many harbor defective or parital viral genomes (Brown et al., 1979).

Currently two animal models exist for neural latency of HSV. Virus inoculation in the foot pad of mice gives rise to a subsequent infection of the spinal ganglia (Stevens and Cook, 1971). This infection is biphasic. A primary acute infection occurs during which high titers of virus are found in spinal fluid and tissues. Virus titers then subside and a chronic phase of infection follows. During this period virus may only be demonstrated by maintenance of ganglia explants in organotypic cultures. The chronic period probably most closely mirrors the quiescent periods in many, but the mouse is not clearly known to undergo recurrence. Intraocular inoculation of rabbits leads to viral spread and involvement of the trigeminal ganglia (Stevens and Cook, 1972; Goodpasture 1929). The infected globe will thereafter undergo periods of quiescence and recurrent infections.

Several parallels can be drawn between chronically infected murine neural tissue and latently infected human ganglia. Only the spinal ganglia associated with sciatic nerves, innervating the site of inoculation, harbor HSV. The virus can be demonstrated by immunofluorescence and electron microscopy after explanation and in vitro maintenance in

organ cultures. Cultures of the sciatic nerve trunk, thoracic spinal cord and medulla oblongata do not yield virus. Persistently infected mice also possess neutralizing antibodies to HSV (Stevens and Cook, 1971). The lack of virus-specific products during the chronic periods suggests that the HSV genome may exist as a subviral unit in ganglionic cells.

Searches for products of viral replication in latent tissue confirm the hypothesis that few ganglionic cells are directly involved with persistence. In chronically infected mice less than 0.1% of the ganglionic cells will act as infectious centers for the virus and those cells positive in fluorescent antibody assays are in the great minority (Walz et al., 1976; Stevens and Cook, 1972). The presence of HSV-specific thymidine kinase activity in the absence of late viral protein markers may indicate an early termination of viral replication in chronically infected murine ganglia (Yamamoto et al., 1977). It is also possible that some species of mRNA are very stable or that transcription continues from "remnants" of the viral genome in some cells.

HSV initially inoculated into the mouse via the ear can be stimulated to reactivate through localized minor trauma to the ear (Hill et al., 1975). During the quiescent periods in this recurrence model, virus is absent from the peripheral tissue, but can be found in the 2nd, 3rd and 4th cervical ganglia. Several forms of stimuli are effective: UV light, cellophane tape, and stripping or swabbing the ear with xylene. All such agents produce a localized inflammatory response and provoke increased levels of prostaglandins within the skin. It is uncertain what role these factors may play during recurrence. It is clear that the effective agents do induce changes within the ganglia as evidenced by increases in infectious virus and tritiated thymidine uptake within the ganglia 2 to 4 days following trauma to the ear (Hill et al., 1975).

Similar observations have been made in HSV latently infected rabbits. In this model, viral spread may be clearly followed from the site of inoculation to the peripheral nervous system and then to the central nervous system in individuals where meningitis and encephalitis ensue. At each level of spread the infection is first manifest in neurons and then adjacent supportive cells, such as Schwann cells or satellite cells (Goodpasture 1929; Baringer et al., 1974). The rate of viral spread within the nerve trunk approaches that of retrograde transport of proteins within the ganglia (Stevens 1975). The kinetics of viral infection and the presence of virus in intra-axonal vesicles has prompted investigators to propose that intra-axonal transport is the principal mode of viral spread (Cook and Stevens, 1973; Kristensson et al., 1974; Baringer 1975). Seventy percent of the trigeminal ganglia in latent rabbits yield virus upon explant while cultures of the conjunctiva, lacrimal gland, cornea, iris, fifth cervical nerve and brain tissue are negative. Immunoflourescent antibody studies on serial sections of rabbit trigeminal ganglia show 1/500 neurons infected with HSV during the first week following corneal inoculation. This ratio of immunopositive to immunonegative neurons falls to approximately 1/2000 to 1/5000 by 20 weeks following inoculation. At this later date the yield of infectious virus from explanted organ cultures is still on the order of 90% (Racjani 1978).

These findings closely parallel the observations of Baringer and Sworeland (1973) in man. Lastly, single abnormal ganglion cells surrounded by a cuff of mononuclear cells may be seen infrequently as late as 11 months post infection. The nuclei of such cells contain HSV particles and their cytoplasm is highly vacolated with virions contained within intra-axonal vesicles (Baringer 1974). It is unclear whether

such cells are the product of the original infection or remnants of a recent recurrence. The mass of this evidence argues that the ganglionic neuron serves as the site of viral latency. The findings also suggest that latency is not a totally "statis" event but is "dynamic" to the extent that, with even long periods of clinical quiescence, a few neurons continue to act as infectious centers within the ganglia.

Although recurrence appears to be a natural phenomenon in rabbits, it can also be artificially induced. Mechanical stimulation is usually achieved through neurosurgical manipulation of the trigeminal nerve root. Following such treatment, virus can be isolated from the tears of 80% of the animals within 48 hours (Nesburn et al., 1976). These findings closely parallel the observations made by Carton and Kilborne (1959) and Pazin et al (1978) in man.

In situ hybridization of HSV specific complementary RNA probes to latent murine ganglia genetic material show viral nucleic acid sequences in a small minority of the neurons (Cook et al., 1974; Zur Hausen and Schulte-Holthausen, 1975). Analysis of hybridization experiments by Puga et al (1978) demonstrates approximately 0.1 genome equivalents of HSV DNA per cell in such ganglia. The quantity of viral RNA fell below the limits of detection during the chronic period. The techniques employed in this work were sensitive enough to detect 1 genome equivalent of viral RNA per 2000 cells but at this sensitivity as many as 400 RNA copies per cell would go undetected.

Finally, using a nick translated ^{3}H-labeled HSV DNA probe, viral specific RNA sequences can be demonstrated in human ganglia sections with in situ hybridization (Galloway et al., 1979). Such sequences may be found in the sacral, thoracic and lumbar ganglia, confirming previous

observations that an individual could be latent at multiple ganglionic sites. In each case only 0.4-8.0% of the ganglionic neurons possessed HSV specific nucleic acid sequences.

Molecular Biology of HSV

The infectious HSV particle has evolved a rather complex architecture. A 1000 Å nucleocapsid of acosahedral symmetry encloses the double stranded linear DNA chromosome and various phosphoproteins of the viral chromatin (Russell et al., 1962; Becker et al., 1968; Spear and Roizman, 1972). This core is itself encompassed by a loose matrix of glycoproteins and carbohydrates of viral origin. Ultimately, the entire structure is surrounded by a lipid envelope, giving the infectious virion a diameter of roughly 1800 Å. Quantitatively the mature particle is comprised of approximately 70% protein, 22% lipid, 7% nucleic acid and 2% carbohydrate (Scott and Tokumaru, 1964; Callaghan et al., 1976). Although the viral genome has the potential to encode and modify through methylation or glycosylation all known virion associated proteins, the lipid moiety of the envelope appears to be principally derived from the phospholipids of the infected cell's nuclear membrane (Watson et al., 1973; Roizman et al., 1974). All known protein species associated with the envelope arise from either virus specified de novo synthesis or modification of cellular proteins (Kaplan 1973; Spear 1972).

Genome Size and Composition

The DNAs extracted from sucrose gradient purified virions of HSV-1 and HSV-2 differ in bouyant density by 0.002 g/cm^3 (Goodheart et al., 1968; Kieff et al., 1971). Goodheart et al (1968) and Plummer et al (1970) determined these densities to be 1.725 g/cm^3 for HSV-1 and 1.727

g/cm^3 for HSV-2. Similar estimates were obtained by Graham (1972) using E. coli DNA as a density reference. These values predict a G + C ratio of 66.3% for HSV-1 and 68.4% for HSV-2 (Goodheart et al., 1975). Kieff et al (1971) established 1.726 and 1.728 g/cm^3 for the respective bouyant densities in CsCl isopycnic gradients using SPO1 DNA as a marker. The resulting 67% and 68% G + C ratios were confirmed by the finding of a 1°C difference in the Tm values for HSV-1 and HSV-2. While Kieff reported the Tm of type 1 to be 83°C and type 2 to be 84°C, Graham et al (1972) found only a 0.1°C difference in Tm values of 82.6° and 82.7°C.

Both serotypes of HSV have a sedimentation rate in neutral sucrose density gradients of 55 S with a co-responding molecular weight of $99\pm5x10^6$ daltons (Kieff et al., 1971; Sheldrick and Berthelot, 1974). The contour length for the linear duplex molecule, based on EM studies, is approximately $100x10^6$ daltons (Sheldrick and Berthelot, 1974; Wilkie, 1973). The estimated molecular weight of HSV-1 circles is $94\pm3x10^6$ daltons; if one considers a 3% redundancy in their terminal overlap this would suggest a value for the linear molecule of $97\pm3x10^6$ daltons (Grafstrom et al., 1974). In a comparative analysis of restriction endonuclease fragments from 80 isolates of HSV-1 Buchman et al (1978) found a variation of only 2% in the determination of overall molecular weight between isolates.

The similarities between HSV-1 and HSV-2 are apparently not limited to a cursory examination of molecular weight and composition. Liquid and filter hybridization analysis of their DNAs suggests an 85% homology over 46% of their genomes; the remaining 56% of their respective genomes is highly variable with little homology (Kieff et al., 1971). Nearest - neighbor analysis for the two gives virtually identical frequency patterns.

However, a 2.2% difference in G + C ratios would predict a mismatch of at least 3850 - base pairs between serotypes (Subak-Sharpe et al., 1973). Polypeptide mapping studies of intratypic recombinants give strong evidence of a collinearity in gene ordering within HSV-1 and HSV-2 chromosomes. The reported viability of intratypic recombinants and the ability of ts mutants of HSV-1 to complement ts mutant of HSV-2 also suggest little divergence within functionally critical regions of the genomes (Subak-Sharpe et al., 1972; Morse et al., 1977). Such evidence indicates extensive similarity in the gross architecture of the two genomes and argues for a distant but common lineage.

An interesting characteristic of the structure of the HSV chromosome is the presence of single-strand interruptions in the DNA. When alkali denatured DNA from purified virus is sedimented on alkaline sucrose density gradients numerous bands of single-stranded DNA may be observed. These bands correspond to fragments of $7x10^6$ daltons to intact strands $48x10^6$ daltons in weight (Kieff et al., 1971; Wilkie 1973). Frenkel and Roizman (1972) have distinguished 6 classes of fragments, ranging from $10x10^6$ to $39x10^6$ daltons, in denatured HSV-1 DNA. The kinetics of reassociation within the intact size, $39x10^6$ daltons, class indicated this class was composed of elements representing a single unique strand of DNA. In conflict with this interpretation are the findings of Wilkie (1973) which suggested a randomly gapped genome and nonunique intact strands. To date no biological function has been ascribed to the single strand gaps and the controversy over random vs. defined sizes has not been reconciled. Newly synthesized viral DNA has been reported to contain short stretches of RNA (Biswal et al., 1974). It is unlikely that such RNA stretches alone would account for these gaps since the

sedimentation profiles in neutral sucrose gradients are indistinguishable for DNA denatured with formamide versus alkali (Roizman, 1979). The consequences of a randomly gapped genome in the absence of a mechanism for repair are self evident. It can be speculated that abortive transcription due to an early truncation of the template could play a role in viral latency or oncogenic transformation. It is noteworthy that no repair mechanism has yet been clearly demonstrated.

Nucleotide Sequence Arrangement

If following denaturation, single strands of HSV DNA are permitted to reanneal a high degree of self annealing can be observed by electron microscopy. Among those strands that have folded back on themselves three recognizable structures can be identified: a linear form with extensive folding at one end, a single stranded circle with a heavily folded region and a structure consisting of two single stranded circles joined by a duplex region (Sheldrick and Berthelot, 1974). The forming of two single stranded circles connected by a double stranded region is indicative of the presence of two inverted repetitions of the termini within the molecule. In a similar manner duplex circles may be formed after treatment of hative HSV DNA with exonuclease III (Sheldrick and Berthelot, 1974; Grafstrom et al., 1975). Such behavior is consistent with the presence of natural repetitions at the single strand termini. Thus the HSV chromosome is bracketed by direct repeats and consists of two unique gene regions of differential length separated by two tandemly arranged inverted repetitions of the termini. One implication of such a model is that following concatameric replication of the molecule the relative orientation of the two unique gene regions may be inverted through recombination between the external and internal repetitions (Thomas and MacHattie, 1967; Sheldrick and Berthelot 1974).

Partial denaturation mapping of the chromosome confirms this architecture and permits the sizing of the various regions. The long unique gene region comprises 70.2% of the genome while the shorter unique region involves 9.4%. The direct and inverted repeats bracketing the long region each represent 6% of the genome length and can be further divided into two subregions: the a, a' reiteration and the b,b' reiteration, which is found only in the long segment. In like manner repeats associated with the short unique region can be designated a'c' and ca, respectively; comprising 4.3% of the genome. The a,a' sequences are held in common between the long and short gene regions while the c, c' and b, b' inversions are limited to the short and long regions respectively (Wadworth et al., 1975). Partial denaturation studies and subsequent endonuclease digestion mapping further confirm that the long and short components are able to invert in their relative orientations. Thus, as originally predicted by Sheldrick and Berthelot (1974), within a population of HSV DNA four conformations of the molecule can be determined which differ in the relative orientations of the long and short unique regions (Hayward 1975; Delins and Clements, 1976; Wilkie, 1976). The diagram below illustrates this point.

One consequence of such an arrangement is that endonuclease cleavage fragments spanning the L/S junction will be found in a 0.25 molar ratio relative to unique fragments and the terminal fragments will be present in a 0.5 molar ratio. This prediction was confirmed by Hayward (1975) in an analysis of the HindIII and Eco RI digestion fragments of HSV-1. Morse et al (1977) has suggested that another consequence of this genome architecture is that not all conformations may be functionally equivalent. If one aligns the amps of intertypic

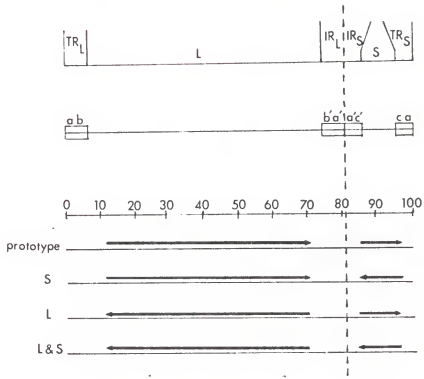

Figure 1. Inversion of HSV DNA

recombinants to give the least number of crossover points, the resulting progeny could only have originated from recombination between parents which were both in the prototype configuration. Such an approach does not take into account the contribution of double cross over events (Morse et al., 1977; Roizman, 1979). To date analysis of recombination frequencies between genetic markers in the long and short regions has shed little light on the validity of this assumption.

Endonuclease Cleavage Map

In addition to the size variation in fragments spanning the L/S joint, arising from inversion of the unique components, two other classes of heterogenity are evident in cleavage maps of both HSV-1 and HSV-2. One type of heterogenity maps solely to the unique regions and appears to define viral strains. The differences within this class arise from the presence or absence of specific endonuclease cleavage sites and are found throughout the long and short components (Skare, 1975; Hayward, 1975; Locker and Frenkel, 1979). In a study of eighty HSV-1 isolates, Buchman (1978) determined 19 out of 60 cleavage sites which could be either present or absent; suggesting the possibility of at least 2^{19} viral strains within the population. At this level of analysis the cleavage maps of HSV-1 and HSV-2 are quite distinct. This first class of heterogenity is less frequent in HSV-2 (Roizman, 1979). The loss or gain of a single cleavage site need not give rise to a new clinically or immunologically recognizable viral strain. Thus the number of immunologically identifiable strains need not approach 2^{19} value. The ability of HSV to remain latent within an individual with intermittent recurrences would provide a natural reservoir for the accumulation of nonlethal mutants within the population. The lower frequency of heterogenity in HSV-2 may simply mirror a smaller reservoir within the populace. The second class of heterogenity maps to the termini of the long unique region (Wagner and Summers, 1978; Skare and Summer, 1977) of HSV. These differences derive from the insertion of a 200bp segment into the terminal reiteration of the L component in single or multiple copies giving rise to a series of minor fragments with a distinct $2x10^5$ dalton increment in size (Wagner and Summers, 1978). Here again a variance between viral strains may occur since a $3.3x10^5$ dalton

increment in size is observed between minor fragments in HSV-1 (F) DNA. In addition, while the minor fragments from the termini of HSV-1 differ by 255 base pairs, those arising from the L/S junction differ by only 125 base pairs (Locker and Frenkel, 1979). Subsequent work with HSV-1 (F) has directly linked the tandem addition through recombination of the 501 base pair 'a' segment to the L termini with the generation of those minor bands (Mocarski et al., 1980). Size variability has also been reported for endonuclease fragments containing the c, c' repeat regions (Lonsdale et al., 1979). This heterogenity can again be traced to the copy number of a 23 base pair repeat present within the c regions. The reiteration can occur in a variable copy number between viral strains, between clones of same viral strain and lastly between the c and c' reiterations of the same strain (Watson and Van de Woude, 1982; Rixon and Clements, 1982).

Transcription

Once a permissive host cell is infected, viral replication rapidly follows in a well-ordered seuqence of events. With uncoating, transcription of defined regions of the viral genome proceeds in a highly regulated temporal manner (Clements et al., 1977; Jones and Roizman, 1979). Transcription of host cell specific RNA (C-RNA) continues at a reduced rate, but synthesis and processing of cellular mRNA is rapidly inhibited, as is the appearance of mature cellular rRNA. This inhibition appears to be coded for by the earliest viral transcripts (Wagner and Roizman, 1969; Kaplan, 1973). For the most part, the reduction in the rate at which mature rRNA appears is brought about by the interference of infection with its maturation; synthesis of the 45 s RNA precursor continues at roughly the same level as that of C-RNA (Wagner and Roizman,

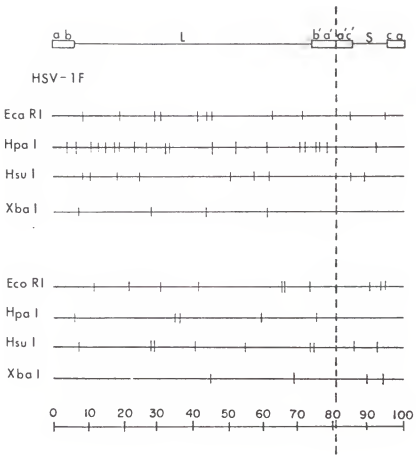

Figure 2. Restriction Map of HSV-1 and HSV-2 DNAs (Roizman, 1979).

1969). However, while there is a stable accumulation of C-RNA within the infected cell, inhibition of host mRNA synthesis is completed to the extent that no de novo synthesis of cellular mRNA can be observed late in infection (Kaplan, 1973). As the rate of host cell DNA synthesis gradually falls off, it is rapidly surpassed by that of nacent viral DNA. By 7 to 8 hours post infection, host DNA is no longer replicated. This inhibition is concomitant with that of host cell protein synthesis and

thus may mirror the loss of proteins specifically required by the cell's replicative machinery (Roizman et al., 1974). The peak rate of synthesis for the earliest viral polypeptides is reached between 2 and 4 hours post infection. The chief function of these proteins is to govern the change over from cellular to viral macromolecular synthesis. There is a stringent requirement for these immediate early polypeptides (α-proteins) prior to the production of a second and larger group of proteins, the early polypeptides (β-proteins), which appear 5 to 7 hours post infection. These β-polypeptides augment the replication of the HSV genome. They also govern a gradual reduction in α-protein synthesis and pave the way for production of the late structural proteins (γ-polypeptides) of the HSV virion (Honess and Watson, 1974; Pereira et al., 1977).

Viral mRNA is transcribed within the nucleus (Wagner and Roizman, 1969), is polyadenylated at its 3' end (Bachenheimer and Roizman, 1972; Silverstein et al., 1976) and carries a 5' cap (Moss et al., 1977). Prior to viral DNA synthesis two classes of viral mRNA, the immediate early and early transcripts, are abundant and are transcribed at least in part by host cell RNA polymerase II (Jones and Roizman, 1979; Clements et al., 1977). Translation of these two classes of transcripts gives rise to the aforementioned α and β polypeptides, respectively. The immediate early mRNA represents approximately 12% of genome and is made in the absence of previous viral polypeptide synthesis. It can be isolated in abundance from the cytoplasm of infected cells treated with cyclohexamide (Jones and Roizman, 1979). These transcripts range from 1,500-5,500 nucleotides in size and map preferentially to the terminally reiterated portions of the genome (Clements et al., 1977, Holland et al., 1979). The second class of mRNA which is abundant before viral DNA

synthesis have a stringent requirement for α polypeptide synthesis prior to its production. The viral DNA polymerase falls within this group. Early transcripts map throughout the HSV genome and the size of their nuclear precursors differs little from that of the polysomal bound population suggesting that their promoters map close to the structural genes (Holland et al., 1979). Together the sequences transcribed prior to DNA synthesis represent approximately 25% of the genome.

Comparative inhibition studies indicate that more than one polypeptide in the α and β groups is involved in regulation of transcription and the transition from α to β to γ protein synthesis. For example canavanine selectively inhibits subsets of these proteins and permits partial transition from immediate early to early and then to late transcription. As with early transcripts, late transcription occurs throughout the genome with approximately 50% of the viral RNA at 6 hours post infection having a length of 5,000 to 10,000 nucleotides. At this time transcription of most early viral RNA continues with γ mRNA species representing approximately 20% of the accumulated viral mRNA.

The complex overlapping hybridization patterns of viral mRNA to the HSV genome suggest that an elaborate series of interlocking controls regulate transcription and translation with multiple RNA species derived from limited regions of the genome and nucleotide sequences common to several messengers. As suggested above the immediate early promoters are compatible with the existing host cell transcriptional machinery yet early promoters appear to have been modified by one or more α viral proteins. The insertion of the 5' promoter region of immediate early (IE) mRNA 3 into sequences upstream from the early thymidine kinase structural gene permits its transcription as an immediate early species

(Post et al., 1981). As with other mammalian DNA viruses both mRNA splicing and overlapping coding regions has been documented. With respect to overlapping coding regions, the 5' termini of two early mRNA's have been mapped to the region of the HindIII cleavage site at 0.586. The regulatory signals for transcription of the smaller 1.2 kb mRNA are found within the larger 5.0 kb transcript. Both mRNAs are unspliced. The 5.0 kb species encodes a 136,000 dalton protein while the smaller 1.2 kb messenger codes for a 38,000 dalton protein. These early mRNAs have a 3' co-terminus (Anderson et al., 1981; McLauchlan and Clements, 1982). Analysis of the co-terminus revealed the sequence AAUAAA with an A + T rich region 3' to the sequence. This is the general format for the 3' termini of all early HSV mRNAs studied to date.

The IE mRNA-4 and IE mRNA-5 share a common 5' sequence. In this instance the promoter and capped 5' termini map to the reiterated sequences flanking the short unique region. Both messages share a common 247 bp leader with a single splice located within the reiterated sequences. A 'TATA' box is located approximately 25 bases 5' to the leader and the common splice appears to function only to remove the introns from a 5' untranslated region (Rixon and Clements, 1982). Since these are the only messages located in this portion of the genome, splicing does not increase the coding capacity of this region. It should be noted that between IE mRNA-3 and IE mRNA-5 is a stretch of 800 nucleotides which is not transcribed. This region may include the origin of DNA replication (Vlanzy and Frenkel, 1981; Watson and Van de Woude, 1982). Lastly, comparison of the 5' terminus of the IE mRNA-5 gene to that of early thymidine kinase gene revealed that the regulatory sequence GGCGATTC was absent in the immediate early gene while present 80 base 5' to the

early transcribed region (Watson, 1982). Eco R1 endonuclease cleavage at this sequence causes reduced levels of thymidine kinase expression (Wigler et al., 1977; McKnight and Gavis, 1980) and the sequence is similar to the RNA polymerase II regulatory signal GGPyCAATCT described by Benoist et al (1980). Thus transcriptional control at the promoter level must distinguish between immediate early, early and late signals and between origins of transcription within the same class; as in the case of the two early promoters described above with a 3' co-terminus.

The rationale for studying the molecular status of the latent herpes genome is in reality twofold. In a general sense, if the latent genome is present as a subviral unit within the cell it provides a well defined gene region of which specific questions concerning gene content and transcription may be asked. In theory, such a genetic system would be subjected to many of the restraints governing the activities of the genes of its eukaryotic host, regardless of whether the viral DNA was integrated or funcitonally an episome. The more direct concern of this study is to approach the question of whether the HSV genome is functionally dormant during its latency or does itself provide some necessary activity. An assay for specific regions of the HSV genome should determine which portions of the viral DNA are most frequently retained in latency while an evaluation of viral RNA will determine which regions of the genome are most frequently expressed. The retention and expression of certain genomic regions common to all HSV latently infected ganglia argue that the virus may play an active rather than passive role during latency.

MATERIALS AND METHODS

Purification of Ganglion Cell DNA and RNA

Teased ganglia preparations were washed 2 times in 1 ml of 1XSSC (150 mM NaCl and 15 mM $Na_2C_6H_5O_7$, pH 7.6), Dounce homogenized and transferred to siliconized 1.5 ml Eppendorf centrifuge tubes. The homogenate was spun at 10,000 rpm for 2 minutes to pellet nuclei from which the DNA was extracted. The resulting supernatant was extracted 2 times with phenol-cresol-chloroform (5:1:1 mixture) and ether extracted 3 times. From the extracted supernatant, total cytoplasmic RNA was precipitated with diethylpyrocarbinol (DEPC) treated ethanol and stored at -20°C. For isolation of nuclear DNA, the pellet was first resuspended in 1 ml 1XSSC, 1% sodium dodecyl sulfate (SDS) and then treated with pronase (1 mg/ml) at 37°C overnight. The preparation was then phenol extracted 2 times, ether extracted 3 times and RNase A treated (20 mg/ml) for 30 minutes at 37°C to degrade nuclear RNA. This was followed by 2 additional phenol extractions. The DNA was then precipitated with ethanol and stored at -20°C. This procedure yielded approximately 26 µg of DNA pre ganglia preparation.

Viral DNA Purification

HSV-1 DNA was isolated as previously described by Holland (1979). Briefly, confluent monolayers of human epidermoid carcinoma cells (HEP-2) were infected with HSV-1 at a multiplicity of 10 PFU/cell and incubated in Eagle's minimum essential medium with 10% calf serum at 37°C (Roizman 1968). Thirty hours post infection the monolayers were scraped into

medium, which was clarified by spinning at 1,000 xg for 15 minutes. The clarified medium was stored on ice and the cell pellet resuspended in cold reticulocyte saline buffer (0.01 m NaCl, 0.0015 m $MgCl_2$, 0.01 M Tris, pH 7.4) and 0.5% Triton. The cells were lysed by 10 strokes in a Dounce homogenizer. The cell homogenate and clarified media were then each spun at 10,000 xg for 15 minutes to remove nuclei and cell debris. The resulting supernatants were pooled and from these HSV-1 virions were pelleted by centrifugation at 33,000 xg for 30 minutes. Virions were resuspended and lysed in 10 mM Tris, pH 7.5, containing 50 mM EDTA, 0.5% SDS and 1% sarkosyl. The lysate was digested overnight at 37°C with pronase at a final concentration of 2 mg/ml (Walboomers and Ter Schegget, 1976). The digest was phenol extracted twice and ether extracted 3 times. HSV-1 DNA was purified by banding twice in an isopycnic ethidium bromide - CsCl gradient. The starting density of CsCl was 1.566 g/cm^3 with 100 µg/ml ethidium bromide and centrifugation was for 48 hours in a Ti50 rotor at 45,000 rpm (Pater et al., 1976).

Partially purified HSV-1 F strain DNA was also received from Dr. Saul Silverstein, Columbia University, New York, NY. These preparations had been treated in the following manner: Nucleocapsids were purified from cytoplasmic extracts of infected Vero cells by velocity sedimentation in sucrose gradients. The virions were lysed in the presence of 0.5% SDS, 0.5% sarcosyl and 10 mM EDTA. The lysate was then extracted with phenol, chloroform and 2% isoamyl alcohol to remove proteins and lipids. The DNA was then precipitated with ethanol and stored (Spear and Roizman, 1972).

Viral DNA preparations received in this form were resuspended in 2XSSC and further purified on CsCl density gradients. A single viral band was obtained at a density of approximately 1.728 g/cm^3 which was well separated from contaminating, cellular DNA and RNA in the pellet (see

Figure 3). The HSV-1 DNA was not highly fragmented as shown by ultracentrifugation on 5-20% alkaline sucrose gradients using ^{32}P-labeled form II SV40 as a size marker (see Figure 4) (Kieff et al., 1971; Pignatti et al., 1979).

Preparation of Cloned DNA

Two clones of the reiterated junction between the long and short unique regions of HSV-1 DNA designated pRB104 and pRB115 were obtained from B. Roizman, University of Chicago, Chicago, IL. pRB104 was generated by the insertion of the Bam H1 fragment SP_2 of F strain HSV-1 into the Bam H1 site in the tetracycline resistance gene of the Escherichia coli plasmid pBR322; pRB115 was derived in like manner but contained the Bam H1 fragment SP_1 of F strain HSV-1 (see Figure 5). The plasmids were provided in E. coli strain C600SF8 (Post et al., 1980). Stocks were maintained in 50% glycerol at -70°C. Prior to growth, single colony isolates were selected for ampicillin resistance, and grown overnight at 37°C in Luria broth with 100 mg/l ampicillin. Generally 20 mls of the overnight culture were used to inoculate 2 liters of broth for the production of DNA stocks. The cultures were induced with the addition of 200 μg/ml chloramphenicol 15 hours before harvest.

Upon harvest the cultures were centrifuged at 10,000 xg for 15 minutes to pellet the bacteria. The bacterial pellet was washed once in a solution of 10 mM EDTA and 50 mM Tris, pH 7.5 and then resuspended in a solution of 25% sucrose, 10 mM EDTA, and 50 mM Tris HCl, Ph 7.5. All subsequent extractions were conducted at 4°C. The bacterial cell wall was digested with egg white lysozyme (500 μg/ml). After 20 minutes in the presence of lysozyme, (1 μg/ml) DEPC was added to inhibit breakdown of nucleic acids and the cells were gently lysed in 0.5% Triton. Low molecular weight DNA was extracted in the manner described by Hirt (1967).

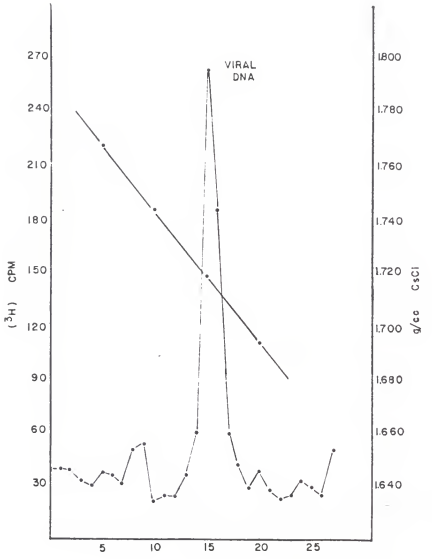

Figure 3. CsCl isopycnic gradient purification of HSV DNA

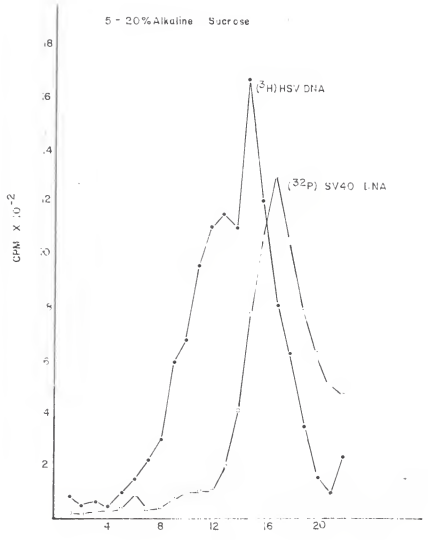

Figure 4. Alkaline Sucrose Gradient analysis of HSV DNA

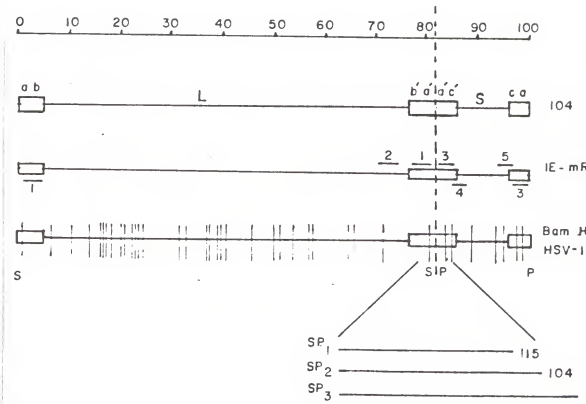

Figure 5. Origin of pRB104 and pRB115 DNAs

The Hirt supernatant was brought to 0.5 M NaCl and polyethylenegycol (PEG) 6000 was added to a final concentration of 5%. The plasmid DNA was permitted to precipitate overnight at 4°C. The resulting pellet was isolated by centrifugation and resuspended in 50 mM Tris, pH 7.6 and 10 mM EDTA; excess PEG was removed by chloroform-phenol extraction and the nucleic acids were ethanol precipitated in 250 mM sodium acetate, pH 4.7, at -20°C. Residual RNA in the resultant pellet was degraded by RNase A treatment and form I plasmid DNA was subsequently isolated by ultracentrifugation on CsCl-ethidium bromide isopycnic gradients (LePecq, 1970).

DNA Restriction

Viral DNA (3 μg) was digested with either EcoRl, HindIII or HpaI endonuclease in a 50 μl reaction mixture at 37°C for 2 hours; 1 unit of enzyme per μg DNA was added per hour. The enzymes were obtained from Bethesda Research Laboratories, Inc., Rockville, MD. Reaction buffers used were in accordance with the instructions provided by the supplier. Ganglionic DNA was digested with EcoRI or HindIII in a 100 μl reaction under the above conditions.

To develop a fine structure restriction of the BamHl fragment spanning the joint region of HSV-1 DNA, cloned viral DNA was digested by BamHl, AluI, BstEll, TaqI, HincII, AvaI and SmaI endonucleases. All cleavage reactions were conducted in accordance with the instructions provided by Bethesda Research Laboratories, Inc. BstEll and TaqI endonuclease digestions were carried out at 60°C: all other digestion reactions were done at 37°C.

Gel Electrophoresis

Restriction enzyme digestions were phenol extracted and then electrophoresed on 0.5% or 1% agarose vertical slab gels at 2 V/cm. Viral DNA

was run on 40x15x3 cm 1% slabs with 1 cm wells, each receiving 500 ng HSV-1 DNA in 10 µl of loading solution. Stock loading solution contained 0.05% bromphenol blue, 0.05% xylene cyanole and 50% glycerol in 0.01M Tris, pH 7.4. This was diluted 1 to 3 with the digests prior to loading. Digests of cloned viral DNA were electrophoresed in the same manner. Cellular DNA was run on 14x12x3 cm 0.5% agarose slabs with 1 cm wells; 10 µg of DNA was loaded as above per well. The running buffer was continuously circulated and contained 5 mM sodium acetate and 1 mM EDTA in 40 mM Tris, pH 7.8.

For size determination and mapping, the digests of cloned viral DNA were also electrophoresed on 6% acrylamide - 0.15% bisacrylamide or 12% acrylamide - 0.30% bisacrylamide gels. DNA (3 µg) was loaded as above per 5 mm well on 40x15x3 cm gels and 1 µg per 5 mm well on 40x15x1.5 cm vertical slabs. The running current was 3 volts/cm. The continuously circulated buffer was 40 mM sodium acetate and 2 mM EDTA in 50 mM Tris, pH 7.8.

Isolation of Endonuclease Cleavage Products

Endonuclease cleavage products were isolated and purified following agarose gel electrophoresis in the manner described by Finkelstein and Rownd (1978). Ethidium bromide stained gels were illuminated with a UV lamp and the fragment band sliced out. Gel slices were finely ground and suspended in 200 µl of 100 mM Tris, pH 5.95. Agarose (Calbiochem-Behring LaJolla, California) was added to a final concentration 0.5 µg/µl and the suspension was incubated for 2 hours at 37°C. The degraded agarose was removed by centrifugation at 15,000 xg for 10 minutes. Following two successive phenol and ether extractions the DNA was concentrated by ethanol precipitation.

Smaller cleavage fragments were extracted from 6% acrylamide gels, following the method devised by Maxam and Gilbert (1980). Gel slices were minced and suspended in a solution of 500 mM ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% (wt./vol.) sodium dodecyl sulfate and 10 µg/ml yeast tRNA. This slurry was incubated at 37°C for 10 hours and then the residual acrylamide was removed by filtration through glass wool. The filtrate was phenol extracted once, ether extracted once and then the extracted DNA was concentrated by ethanol precipitation.

Bulk purification of cleavage fragments was done by affinity chromatography (Bünemann and Müller, 1978). Generally 200 µg of digested DNA were loaded onto a malachite gree substituted bisacrylamide gel (Beohringer Mannheim Biochemicals, Indianapolis, IN) column in the presence of 10 mM sodium phosphate and 1 mM EDTA, pH 6.0. The fragments were eluted through a 0-2 molar gradient of sodium perchlorate in the same phosphate buffer (see Figure 6). The sodium perchlorate was removed from the eluate by dialysis against 10 mM Tris, 1 mM EDTA; pH 7.0. The DNA was the concentrate from the dialyzed eluate by ethanol precipitation.

Mapping Approach

Endonuclease digestion maps for pRB104 and RB115 were determined by two methods: double digestion mapping and partial digestion mapping. Where applicable, new sites were mapped relative to known sites in reactions where two or more digestions were done to completion before gel electrophoresis of the fragments. All reactions and subsequent gel fractionations were carried out as previously discussed. In this manner the digestion sites for BamHl, BstEll, HincII, Alul, and Taql were confirmed and ma-ped relative to one another. The size of the endonuclease

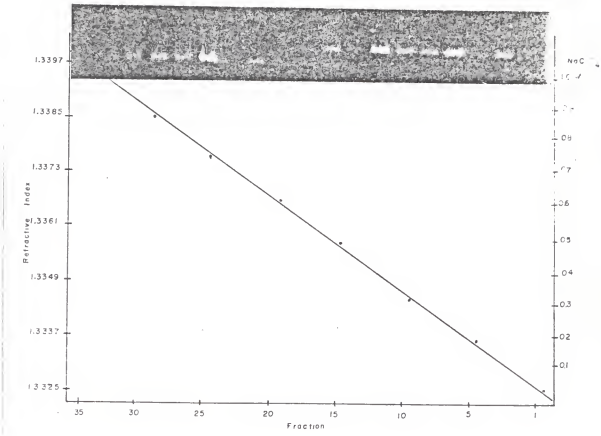

Figure 6. Affinity Chromotography of BamH1 digest of pRB104

cleavage fragments was determined by comparison to known digestion standards of adeno-associated virus (AAV) DNA, lamda phage DNA, or pBR322 DNA, which were run in parallel wells on the same gels (Berns and Hauswirth, 1978; Cheung et al., 1980). The numerous cleavage products arising from restriction by either AvaI or SmaI were oriented relative to the AluI and TaqI sites through partial digestion reactions in the manner described by Smith and Birnstiel (1976). End labeled AluI or TaqI fragments were isolated and partially digested in the presence of 1 unit/μg DNA of AvaI or SmaI endonuclease under standard conditions. Aliquotes were withdrawn from cleavage mixture and prepared for gel electrophoresis after 5 minutes, 10 minutes, 20 minutes, 40 minutes and 80 minutes of reaction time.

Nick Translation

1 μg HSV-1 F strain DNA was incubated for 45 minutes at 15°C in a 50 μl reaction mixture containing 30 mMdGTP, 30 mMdTTP, 30 mMdATP (sigma), 100 μCi[α - ^{32}P]dCTP (400 Ci/m mole; New England Nuclear), 5 mM $MgCl_2$, 4 units DNA-polymerase I, 10 mM β-mercaptoethanol, 0.05 mg/ml bovine serum albumin, 50 mM Tris, pH 7.8, and 4.5 units of DNA-polymerase I (Boehringer Mannheim Biochemicals, Indianapolis, IN). The reaction was stopped by the addition of 100 μl of a solution containing 100 mM EDTA, 100 μg sonicated calf thymus DNA and 10 mM Tris, pH 7.8. The mixture was extracted with phenol and ether. Finally free [α - ^{32}P]dCTP was separated from labeled DNA by passage through a Sephadex G75 column (1cmx10cm). This procedure usually yielded a viral probe with a specific activity of approximately $3x10^8$ cpm/μg. Cellular DNA was labeled in the same manner except that the reaction volume was increased to 100 μl and 10 μg DNA was labeled per reaction in the presence of 0.5-2 ng activated DNase I.

DNase I was activated by incubating the enzyme for 2 hours at 0°C in a solution containing 10 mM Tris, pH 7.6, 5 mM $MgCl_2$ and 1 mg/ml bovine serum albumin. DNase I was obtained from Worthington Biochemical Corporation, Freehold, NJ and stock solutions of 1 mg/ml in 0.01 n HCl were stored at -20°C (Rigby et al., 1977).

Iodination of Cytoplasmic RNA

Ethanol precipitated cytoplasmic RNA was resuspended in 50 µl of DEPC treated distilled water and transferred to a 1.5 ml Eppendorf centrifugation tube containing 4 mCi ^{125}I sodium iodide. To this, 20 µl of 0.2M sodium acetate (pH 4.7) and 5 mM thallic chloride was added. The tube was sealed and incubated at 70°C for 20 minutes. ^{125}I sodium iodide with a specific activity of 350-600 mCi/m mole was purchased from Amersham/Searle Corporation, Arlington Heights, IL. Thallium chloride was purchased from ICN Pharmaceuticals, Incorporated, Plainview, NY. After incubation the reaction was chilled and stopped by the addition of 20 µl of 0.02 Mβ-mecaptoethanol, 1 M sodium phosphate, pH 6.8. The solution was heated to 70°C for 15 minutes and 50 µg of yeast tRNA was added as carrier. Labeled RNA was separated from free ^{125}I by passage through a Sephadex G75 column (1cmx10cm) (Tereba and McCarthy, 1973).

5' End Labeling Reactions

The 5' ends of endonuclease digestion fragments were labeled with [γ - ^{32}P]dATP in the presence of T_4 polynucleotide kinase (Richardson, 1966). The 5' terminal phosphate of the polynucleotide was first removed by treatment bacterial alkaline phosphatase in 10 mM Tris, pH 7.4 and 100 mM NaCl at 60°C for 30 minutes. To halt the reaction 5 mM EDTA was added and the solution was phenol extracted twice then ether extracted

3 times. The DNA was then precipitated with ethanol and dried under vacuum. The DNA was redissolved in a solution containing 50 mM Tris, pH 9.5, 10 mM $MgCl_2$, 5 mM dithiolthreatol, and 50% glycerol. To this was added 100 mM spermidine and 20 units T_4 polynucleotide kinase. The resulting solution was mixed with 200 μCi of dried [γ -^{32}P]dATP and allowed to react at 37°C for 30 minutes. To stop the reaction 5 mM EDTA was added and the solution was dialyzed against 10 mM Tris, pH 7.4, 5 mM EDTA, and 1 M NaCl to remove unreacted [γ -^{32}P]dATP. The bacterial alkaline phosphatase was purchased from Worthington Biochemical Corporation, Freehold, NJ; the Kinase was obtained from Bethesda Research Laboratories, Inc., Rockville, MD; and the [γ -^{32}P]dATP was obtained from Amersham, Arlington Heights, IL. Generally 50-100 μg of DNA were labeled in a 50 μl reaction volume to a specific activity of 10^5 cpm/μg of DNA.

The large "A" fragment of E.coli polymerase I was used to 3' end-label various digestion fragments. For this purpose the 50 μl cleavage reaction mixture was blown down to approximately 20 μl and 20 μCi of [α -^{32}P]dCTP or [α -^{32}P]dATP with 0.9 units of the Klenow polymerase fragment (New England BioLabs, Beverly, MA) were added. Labeling was done at 37°C for 30 minutes. To half the reaction the sample was placed at 60°C for 10 minutes, then 20 μg of sonicated carrier DNA was added and the sample was ethanol precipitated.

Southern Blots

DNA restriction fragments were transferred from agarose or polyacrylamide gels to nitrocellulose filters in the manner described by Southern (1975). Gels were first stained in a 1 μg/ml ethidium bromide bath, visualized under UV light and photographed. The right corner of the gel

and the furtherest points of migration were notched to facilitate future orientation of the gel. The DNA fragments were then denatured in situ by soaking the gel for 20 minutes in a 1 M KOH solution. The KOH wash and gel were then tritrated to neutrality with 1M HCl-1M Tris. The gel was then aligned on a prewashed nitrocellulose filter for transfer. The nitrocellulose filter was prewet by washing for 20 minutes in a 5XSSC bath. The DNA was eluted from the gel with 10XSSC. For transfer from a 1% or greater agarose gel, the gel was placed on a paper wick soaked in 10XSSC, overlaid with the nitrocellulose and absorbant material. This arrangement permitted a higher volume of 10XSSC to pass through the gel and enhanced transfer of higher molecular weight fragments. For transfer from agarose gels of less than 1% and polyacrylamide gels, a slightly different procedure was used in order to decrease diffusion of the DNA bands during transfer. The filter was again sandwiched between the gel and absorbant material but arranged in the reverse order so that transfer was in a downward direction. The gel was overlaid with a paper wick which was periodically wet with 10XSSC. Thus a decreased volume of 10XSSC was passed through the gel and diffusion was diminished. After transfer the nitrocellulose filter was dried at 80°C for 2 hours under vacuum.

Hybridization Reaction

Hybridizations were carried out in sealed plastic bags containing the filter and a hybridization mixture composed of the following: 50% formamide, 5XSSC, 0.08% polyvinylpyriodate, 0.08% bovine serum albumin, 0.08% ficoll, 0.5% SDS, 0.02 M Tris (pH 7.4), 50 µg/ml sonicated calf thymus DNA as a carrier and either ^{32}P or ^{125}I-labeled probe at a concentration of at least 10^6 cpm/ml. All blots were prewashed with the

hybridization mixture minus the probe at 37°C for 2 hours prior to the hybridization. Hybridization was carried out in the presence of the complete mixture for 72 hours at 37°C. The blots were then removed and washed twice to remove nonspecifically bound label, with the hybridization mixture minus carrier and probe for 30 minutes at 37°C. The blots were then rinsed twice with 2XSSC and 0.5% SDS in 50% formamide. The blots were washed twice in 1XSSC and 0.5% SDS in 50% formamide for 30 minutes at 37°C (McConaughy et al., 1969; Tereba and McCarthy, 1973). Finally, the blots were washed overnight at 65°C in 1XSSC and 0.5% SDS, with 4 changes of the wash.

On occasion the hybridization was carried out at 65°C. In these instances the reaction and washes were done as described above at 37°C but in the absence of formamide. Attempts were made to increase the sensitivity of the hybridization by adding 10% dextran sulfate to the hybridization reaction (Wetmur, 1975). The findings were inconsistent due to variable nonspecific background on the autoradiographs of such experiments.

Following the hybridization and washings the blots were dried and then autoradiographed with Kodak X-Omatic regular intensifying screens and Dupont Cronex 4 X-ray film at -70°C for 3-21 days.

RESULTS

Experimental Approach

As stated previously, the general aim of this research is to illuminate the molecular status of the HSV genome in latently infected human trigeminal ganglia. Specifically the studies are designed to

1. Detect HSV DNA and RNA sequences in human ganglia.
2. Determine the extent of the viral genome present through hybridization of HSV DNA to nitrocellulose paper bound ganglia DNA.
3. Determine the extent of viral DNA expressed as cytoplasmic RNA through mapping of ganglia RNA hybridized to nitrocellulose paper bound viral DNA.
4. Correlate genome presence with the expression of a humoral immune response to HSV antigens in immuno-competent patients as measured by complement fixation.
5. Evaluate the manner of viral genome latency.

To achieve this end the following experimental approach was taken. Total cellular DNA and cytoplasmic RNA were extracted from trigeminal ganglia. Three different experimental routes could then be carried out. The DNA was either radiolabeled with [α - ^{32}P]dCTP by nick translation (Rigby et al., 1977) or it was digested with a restriction endonuclease, gel electrophoresed and then transferred and immobilized on nitrocellulose paper by the blotting method of Southern (Southern, 1975; Wahl et al., 1979) (see Figure 7). Radiolabeled ganglia DNA was subsequently

hybridized to EcoRl digests of HSV DNA immobilized on nitrocellulose paper under conditions favorable for formation of DNA-DNA hybrids. The reverse experiment was also done using nick translated [α - ^{32}P]dCTP labeled HSV DNA as a probe and hybridizing with endonuclease digested ganglia DNA.

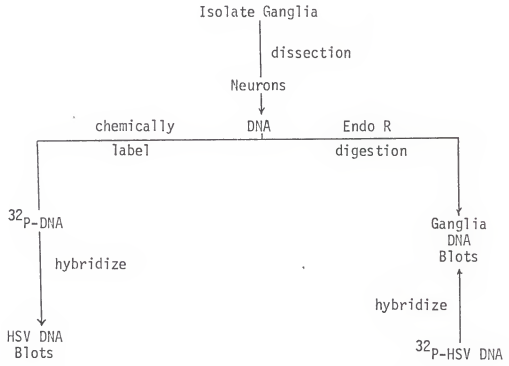

Figure 7. DNA/DNA Hybridization Procedure

The third procedure entailed hybridizing ^{125}I-labeled total cytoplasmic RNA from ganglia to immobilized restriction endonuclease digests of HSV DNA (Tereba and McCarthy, 1973) (see Figure 8). In all these approaches viral DNA or RNA sequences complementary to immobilized sequences were detected by autoradiography at -70°C for 1-21 days. The pattern of radioactive bands was compared to control HSV DNA run in parallel experiments.

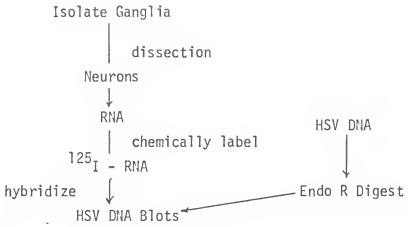

Figure 8. RNA/DNA Hybridization Procedure

Controls

The binding of radiolabeled ganglion DNA or RNA to nitrocellulose bound viral DNA permits the detection of specific genome regions of HSV DNA sequences in neural tissue. This is a considerable refinement over standard filter hybridization methods or *in situ* methods where only hybridization to the whole genome is monitored and where a consideration must be given to high backgrounds due to nonspecific binding of the probe.

The selectivity of the assay is still largely a product of the purity of the known viral DNA preparation. To assay this purity HSV-1 F strain DNA was nick translated and hybridized to EcoRl endonuclease digests of DNA from calf thymus tissue, salmon sperm, human foreskin, Hela M monolayers and Hep 2 cells (see Figure 9). The HSV DNA was prepared by high salt precipitation in the manner described by Pater *et al* (1976). This approach is a modification of the high salt extraction method first devised by Hirt (1967). Nonspecific binding of label occurred with each digestion assayed. This suggests the presence of cellular DNA copurifying within the viral DNA preparation. For this

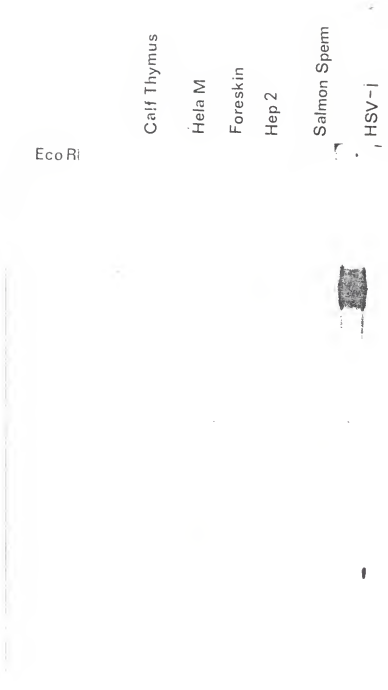

Figure 9. Cellular DNA Controls

reason, only HSV-1 F strain DNA extracted from sucrose gradient purified nucleocapsids and banded twice in CsCl density gradients to remove contaminating cellular DNA and RNA was used throughout (Spear and Roizman, 1972). To further eliminate the possibility of contamination by copurifying cellular nucleic acids, HSV-1 F strain DNA sequences cloned in PBR322 were also used in some of the hybridization studies. DNA isolated from purified nucleocapsids failed to hybridize to cellular DNA in a repetition of the above described assays. In control experiments such a probe also failed to bind to DNA from adenovirus (Ad2), adenoassociated (AAV) virus and Simian Virus 40 (SV40) (see Figure 10). DNAs extracted from thoracic ganglia and connective tissue of patients have been run in parallel reactions with trigeminal gnaglia DNA and failed to hybridize with this probe.

To determine the sensitivity of the hybridization assay reconstruction experiments were run using HSV and AAV DNA. Samples of AAV DNA were digested with either BamHl, EcoRl or AluI and then mixed. Starting with a total of 10 ug of digested AAV DNA serial dilutions of the preparation were then made. To maintain a total of 10 ug DNA in each sample sonicated calf thymus DNA was added as needed. The samples were electrophoresed on a 6% polyacrylamide gel and then transferred to nitrocellulose by the Southern blotting method. Using a nick translated AAV probe with a specific activity of 10^8cpm/ug DNA the sample containing 100 pg of AAV DNA could be visualized by autoradiography after 2 weeks. A 200bp fragment within this sample would represent 5pg of DNA. Because fragments within this size range are visualized the assay is sensitive enough to distinguish 5pg of AAV in 10 ug of calf thymus DNA, a dilution factor of $2x10^6$. Similar experiments using HSV DNA established that

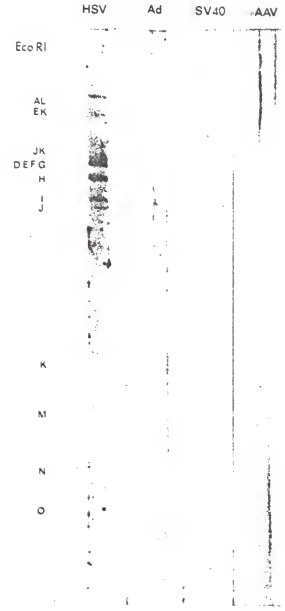

Figure 10. Viral DNA Controls

endonuclease fragments transferred from 0.5% agarose gels to nitrocellulose could be distinguished at a dilution factor of 10^6 of 1pg of HSV DNA in 1 ug of carrier DNA.

Hybridization of Ganglia Nucleic Acids to HSV DNA

The trigeminal ganglia of 18 patients have been assayed for HSV sequences by DNA/DNA or DNA/RNA hybridization. None of the patients manifested clinical evidence of recurrence at the time of death nor was a history of recurrence noted (Table 1).

Table 1. Hybridization of trigeminal nucleic acids to HSV DNA.

Patient	CT titer	Viral DNA*	Viral RNA*
227	32	+	N.D.
228	32	+	N.D.
367	32	+	N.D.
951	64	+	+
883	64	+	N.D.
1072	32	+	N.D.
1034	32	+	N.D.
1082	128	+	N.D.
1089	128	+	N.D.
1033	64	-	-
949	32	-	+
941	<8	+	+
319	<8	-	N.D.
943	<8	-	-
947	<8	-	-
893	<8	-	N.D.
1143	<8	-	-
1110	<8	-	-

*(+) present
(-) absent
(N.D.) not determined

Briefly, the findings from the experimental approaches have been listed in Table 1. The first approach entails the hybridization of a nick translated ^{32}P labeled viral DNA to either EcoR1 or HindIII endonuclease digests of ganglia DNA bound to nitrocellulose filters. The

second is the hybridization of ^{125}I-labeled ganglia cytoplasmic RNA to restriction endonuclease digests of HSV-1 DNA bound to nitrocellulose. Eleven of these patients were sero-positive for HSV. A positive serology here is assessed as a compliment fixation titer of 1:8 or greater. The trigeminal gnaglia from 9 such individuals were shown to contain HSV DNA sequences. Viral DNA was not detected in the remaining two members of this group (949 and 1033) but viral sequences were demonstrated in a preparation of total cytoplasmic RNA from the ganglia of patient #949. Only one of the sero-negative individuals (941) yielded ganglia containing latent HSV DNA and viral RNA was also detected in this case.

Figure 11 is an example of the autoradiographs obtained from hybridization assays involving viral and ganglia DNA. The first lane (A) is a photograph of the ethidium bromide stain of an EcoR1 digest of HSV-1 run as a marker. The second lane (B) is the autoradiograph of labeled viral DNA hybridized to EcoR1 digests of ganglia DNA. Lane C is the photograph of the ethidium bromide stain of the HindIII digested control as it appeared after agarose gel electrophoresis. Lanes D and E are the autoradiographs of labeled viral DNA bound to HindIII endonuclease digests of ganglia DNA. In the experimental reaction with ganglia #228 DNA, extensive binding of the probe is evident in the regions where the larger EcoR1 fragments (MW 8.4-17.5x10^6 dal.) of HSV would migrate as determined by comparison to the controls. Binding is also apparent where the EcoR1 M (MW 2.7x10^6 dal.) fragments are expected to migrate. The viral probe did not hybridize in the region where the EcoR1 K(MW 3.5x10^6 dal.) fragment should be found. EcoR1 K maps at the terminus of the short unique portion of the genome. The fragment may display an altered migration here, or this portion of the viral genome may not be represented in the ganglia. It is of interest to note that

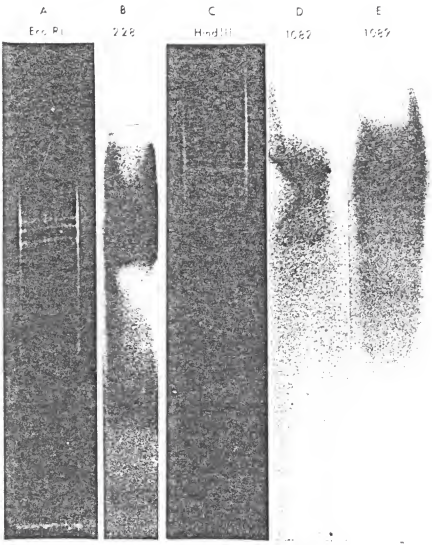

Figure 11. Viral DNA hybridized to Ganglia DNA

ganglia of patient #227 also revealed an altered migration of the EcoR1 K fragment. Unfortunately the HindIII digestion pattern does not permit such an analysis. Patient #228 is representative of the type of findings obtained when a major portion of the HSV genome was demonstrated in the ganglia. In all such cases to date the endonuclease digestion pattern

revealed was consistent with that of HSV-1. In several of the ganglia only part of the HSV genome could be detected; whether the remainder of the genome is absent or those portions are present in too few copies for the assay to reveal is as yet uncertain.

Figure 12 is an autoradiograph of ^{125}I-labeled ganglia cytoplasmic RNA hybridized to an EcoRl endonuclease digest of HSV-1 F strain DNA bound to nitrocellulose paper. To the left of the autoradiograph is a photograph of the cleaved HSV stained with ethidium bromide after agarose gel electrophoresis and prior to transfer to nitrocellulose. Clearly not all regions of the viral genome are represented in the ganglia cytoplasmic RNA. This would not be the case if the ganglia were actively supporting a lytic infection as shown in the HSV control. Further, those fragments present are not uniformly represented in the RNA. For instance, transcripts of EcoRl K appear most abundant in the RNA of patient #951. In this manner the viral RNA transcripts found in several ganglia have been mapped on the HSV-1 genome. Figure 13 summarizes this data.

CsCl Enrichment

To determine whether HSV DNA could be isolated in an episomal form free from host chromatin total ganglia nucleic acid was fractionated on CsCl density gradients. As a control human thoracic ganglia DNA was kinased and run in a parallel gradient. ^{3}H labeled lambda DNA was run as a marker in all gradients and 50 ug of sonicated calf thymus DNA was added to each as a carrier. The gradient profiles are shown in Figure 14.

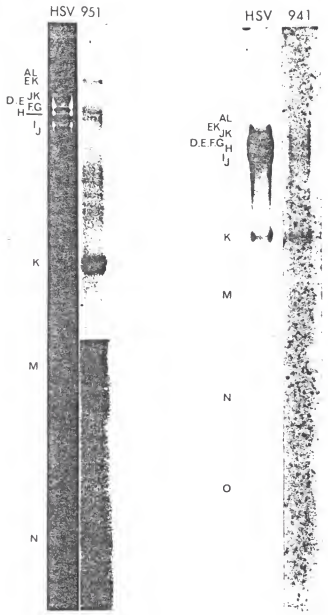

Figure 12. Ganglia RNA Hybridized to Viral DNA

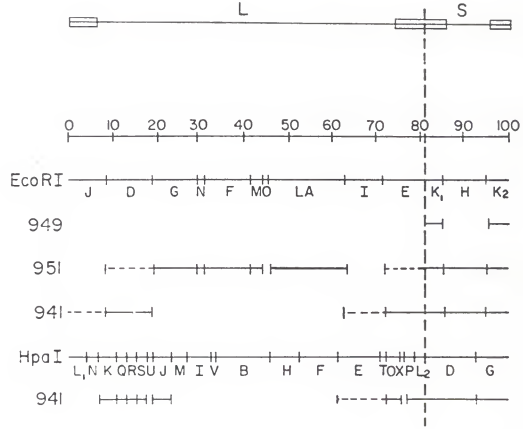

Figure 13. Mapping HSV specific RNA found in the Ganglia

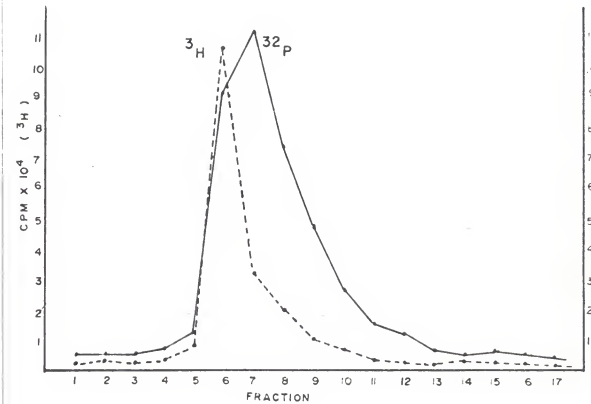

Figure 14. Fractionation of Total Ganglia Nucleic Acid

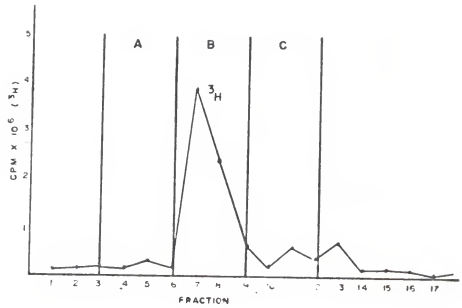

Figure 15. Sectioning of CsCl gradient

Using the lambda marker as a reference the gradients were divided into five segments. Within gradients A, B and C fractions were pooled and the total DNA per section isolated and nick translated. The findings are summarized in Table 2. Approximately 25 ug of DNA from HEP2 monolayers infected with HSV-1 F at an m.o.i. of 1 and harvested at 20 hours post infection was used as a positive control for this assay.

Table 2. CsCl enrichment study

Patient	CF-titer	Probe I	II	III
1168	256	-	-	-
1090	256	-	-	-
1144	128	-	-	-
1143	8	-	-	-
thoracic ganglia	16	-	-	-
HepII	ND	-	-	-
HSV infected Hep2	ND	+	+	-

Mapping the L/S junction of HSV-1 F strain

The immediate early mRNA species of HSV-1 are known to map to the reiterated portions of the genome. This fact coupled with the observation that transcripts mapping to this region were present in all positive ganglia studied to date suggested that this area warranted further analysis. Hybridizations of ganglia RNA to pBR322 clones of the L/S junction were undertaken in order to develop a clearer picture of the degree of transcription. To gain such an understanding it was necessary to first derive detailed restriction digestion maps of the DNA in question.

Two plasmids were used for this purpose, pRB104 and pRB115 (Post et al., 1981; Material & Methods) (see Figure 16). The SP_2 insert in pRB104 is approximately 500bp greater in length than the SP_1 species inserted into pRB115 (Post, 1981). First the DNA was evaluated by single and double or triple endonuclease digestions using BamHl, Pstl, HindII and BstEll. This confirmed the identity of the two plasmids and also allowed us to map a 520bp insertion to the largest BamHl/HindII fragment of pRB115 (see Figure 17). The size markers in these assays were known digests of either pBR322 or AAV DNA. The clones were then screened with various additional endonucleases to determine their digestion patterns. A summary of the restriction enzyme fragment sizes is given in Table 3

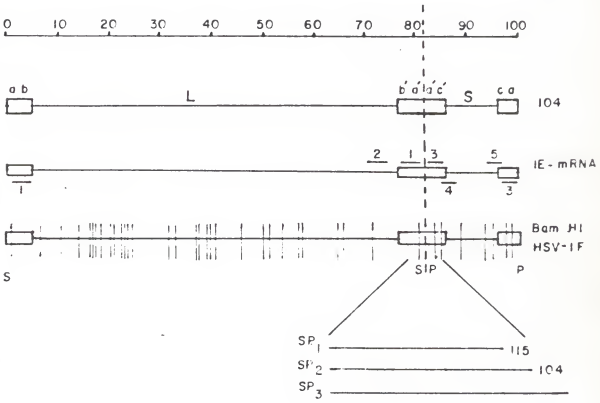

Figure 16. Derivation of Clones pRB104 and pRB115

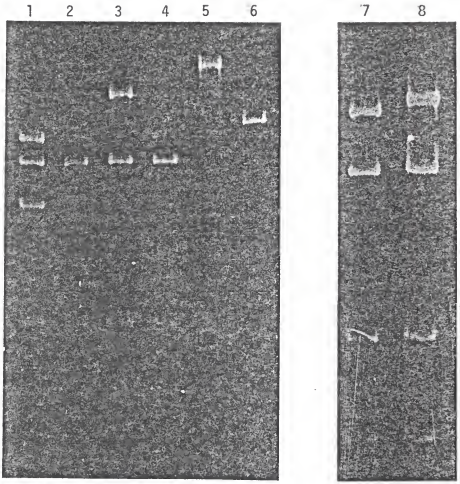

Figure 17. Restriction Digests of pRB115 and pRB104

Lanes:
1. BamH1/Pst1/BstE11 digest of pRB115
2. BamH1/Pst1/BstE11 digest of pBR322
3. BamH1/Pst1 digest of pRB115
4. BamH1/Pst1 digest of pBR322
5. Pst1 digest of pRB115
6. Pst1 digest of pRB322
7. BamH1/HincII digest of pRB115
8. BamH1/HincII digest of pRB104

Table 3. Endonucleases

	BamH1/HindII		BamH1/BstE11		BamH1/BstE11/TaqI		SmaI	AvaI
Clone:	115	104	115	104	115	104	115	115
fragment size (bp)	4600 1230	5200 1240	750 2300	4200 2300	2400 1950 1600	2350 2200 1975	1075 677 577 450 430 365 337 222 140 102 84	890 566 483 453 400 380 336 317 226 206 170 148 138 99 82 55

Enzymes that did not cut: BglII, KpnI, HpaI, XbaI, EcoRI, HindIII, PstI and SalI

and a comparison of the SmaI and AvaI digests of pRB104 and pRB115 are shown in Figure 18. The mean length of the insert in pRB104 was calculated to be approximately 6382 base pairs and that of pRB115 was 5830 base pairs. A comparison of the SmaI and AvaI digestion patterns of the two clones indicates that more than a single insertion event has occurred in the derivation of the SP_1, and SP_2 fragments. The fragment corresponding to the 1075 bp SmaI fragment of pRB115 is only 880bp in pRB104 even though pRB104 contains the larger insert. The 360bp SmaI fragment of pRB115 is overrepresented in pRB104. The 450bp AvaI fragment of pRB115 is overrepresented in pRB104 and the 483bp AvaI fragment of pRB115 is missing in pRB104. The pRB104 AvaI digest also has a cluster of fragments between 330bp and 380bp that are not found in pRB115.

Because of these additional rearrangements the SmaI and AvaI maps of only pRB115 were determined.

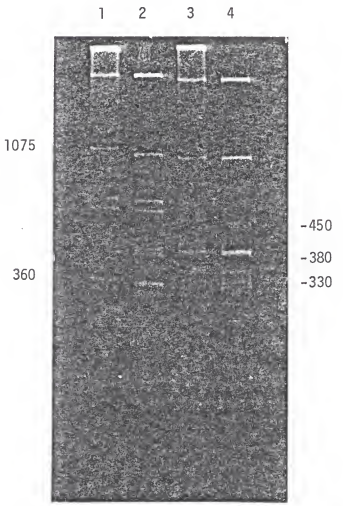

Figure 18. SmaI and AvaI digests of pRB104 and pRB115

Lanes:
1. SmaI digest of pRB115
2. SmaI digest of pRB104
3. AvaI digest of pRB115
4. AvaI digest of pRB104

The SP_1 insert in pRB115 was excised by BamHl cleavage and the insert separated in bulk from the vector by affinity chromatography over a malachite green substituted bisacrylamide gel column. The Sma1 and Aval cleavage maps were determined by partial digestion mapping (Smith and Birnstiel, 1976). The approach was as follows. The DNA was cut with Alul and then 5' end labeled by the T_4 polynucleotide kinase reaction. The DNA was then recut with Taql endonuclease and the digestion fragments separated by agarose gel electrophoresis. The three largest fragments spanning the insert were designated 1, 2 and 3, respectively. These fragments were aligned on the general map using double digestion assays with HincII or BstEll as shown in figure 19a. Partial cleavage mapping was then done using Sma1 and Aval on each of the Alul/Taql fragments. The map was confirmed by first cutting with Taql, labeling and then recutting with Alul; thus generating an overlapping map going in the opposite direction.

Figure 19b shows the generation of the Sma1 map for Alul/Taql fragment 2 and the HincII digestion of fragment 2 and fragment 3. The accompanying figure 19b shows the mapping strategy and the log plot of migration distance versus molecular weight from which the size of the partial Sma1 fragments was determined. The resulting cleavage map of fragment 2 is also shown. Figure 20 shows the partial digestion fragments resulting from Aval cleavage of the Alu[1]/Taql fragments 2 and 3. Sall, EcoRl, BamHl and Alul digests of AAV were used as size markers. The maps resulting from these gels and similar experiments are shown in Figure 21.

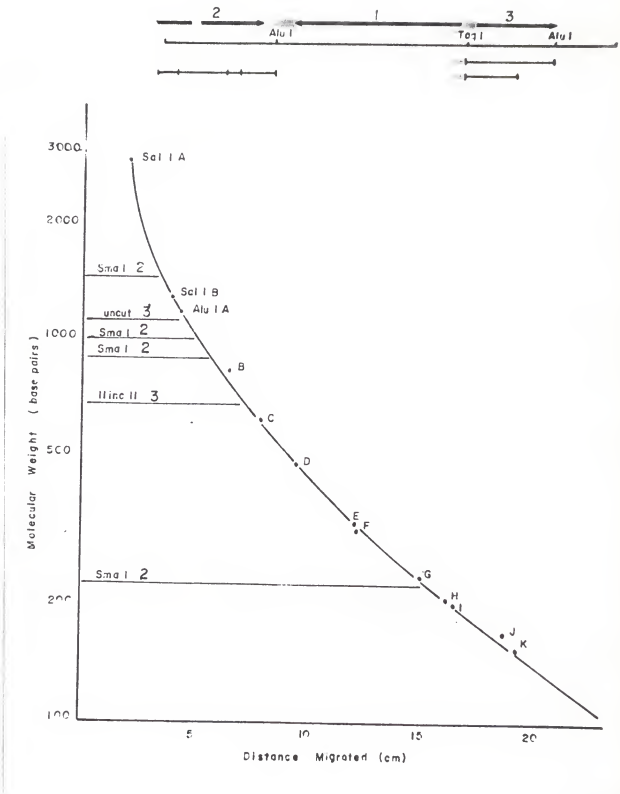

Figure 19a. Strategy for Partial Digestion Mapping of pRB115

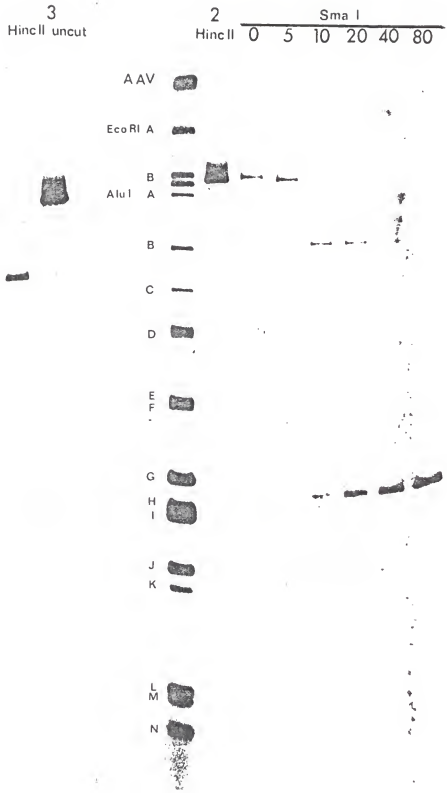

Figure 19b. SmaI partial digestion of pRB115

Figure 20. AvaI Partial Digestion Mapping of pRB115

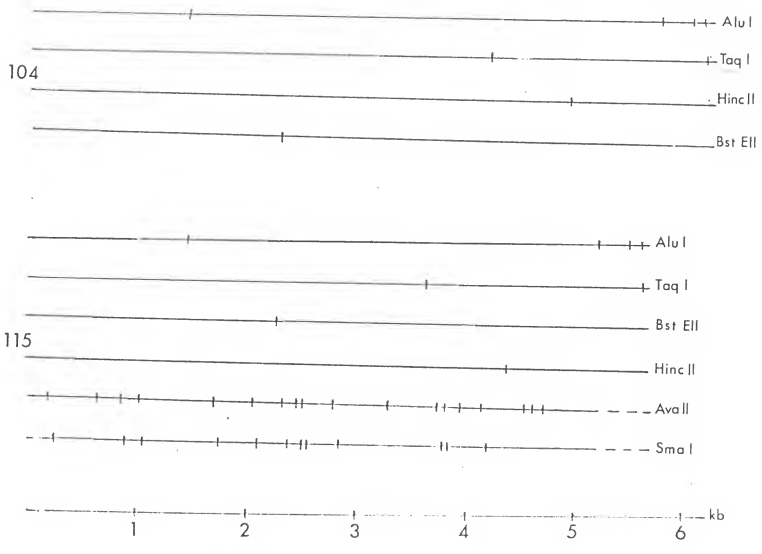

Figure 21. Restriction Digest Map of pRB115

HYBRIDIZATIONS

Hybridization of ganglia RNA to the L/S junction

As stated previously hybridization of total cytoplasmic RNA from ganglia to Southern blots of clone pRB115 of the L/S junction of HSV-1 F was undertaken in hopes of better delineating the level of transcription through this portion of the genome. The ^{125}I-labeled RNA from five sero-positive patients was evaluated in this manner. The nucleic acids from thoracic ganglia and a normal lytic infection were also evaluated as controls. The findings are given in Table 4. As in the

Table 4. Analysis of L/S Junction

Patient	CT titer	Viral DNA*	Viral RNA*
1033	64	-	-
1034	32	+	-
1082	128	+	-
969	16	-	-
1089	128	+	-
thoracic ganglia	16	-	-
HSV infected Hep2	N.D.	+	+

*(+) present
(-) absent
(N.D.) not done

CsCl enrichment studies DNA or RNA from Hep2 monolayers infected with HSV-1 F at an m.o.i. of 1 and harvested at 20 hours post infection were used in the control assays.

Cross Hybridization Experiments

The close biological relationship between AAV, Ad and HSV suggests the possibility of partial genome homology; however previous attempts have failed to demonstrate detectable homology. Using the more sensitive technique of cross hybridization of ^{32}P-labeled AAV, HSV or Ad probes to restriction endonuclease digests of the reciprocal genome immobilized on nitrocellulose filters, we were able to determine limited regions of homology. Figure 22 outlines a typical cross hybridization assay and our findings to date from the hybridization assays are summarized in Table 5.

Table 5. Cross Hybridization Analysis

	BLOT							
	AAV-2	Ad2	Ad5	SV40	HSV-1	Hep2	HelaM	Hela
AAV-2	+	+	+	-	-	-	-	-
Ad2	+	+	+	-	-	-	-	-
Ad5	+	+	+	-	-	-	-	-
SV40	-	-	-	+	-	-	-	-
HSV-1	-	-	-	-	+	-	-	-

(+) homology
(-) no homology

To determine the level of nonspecific hybridization that could occur in our assays, such as that which might arise from long stretches of high G+C content, hybridizations were done with the DNA from the Hep2, HelaM and Hela suspension cell lines. The Ad5, Ad2, and AAV2 virus stocks were grown in Hela suspension cultures. The HSV-1 virus was grown in either Hep2 or HelaM monolayers. Thus these controls would also allow us to detect any cellular DNA which might be copurifying with viral DNA during its preparation. Our findings were consistantly negative although the controls were hybridized under several degrees of stringency as were

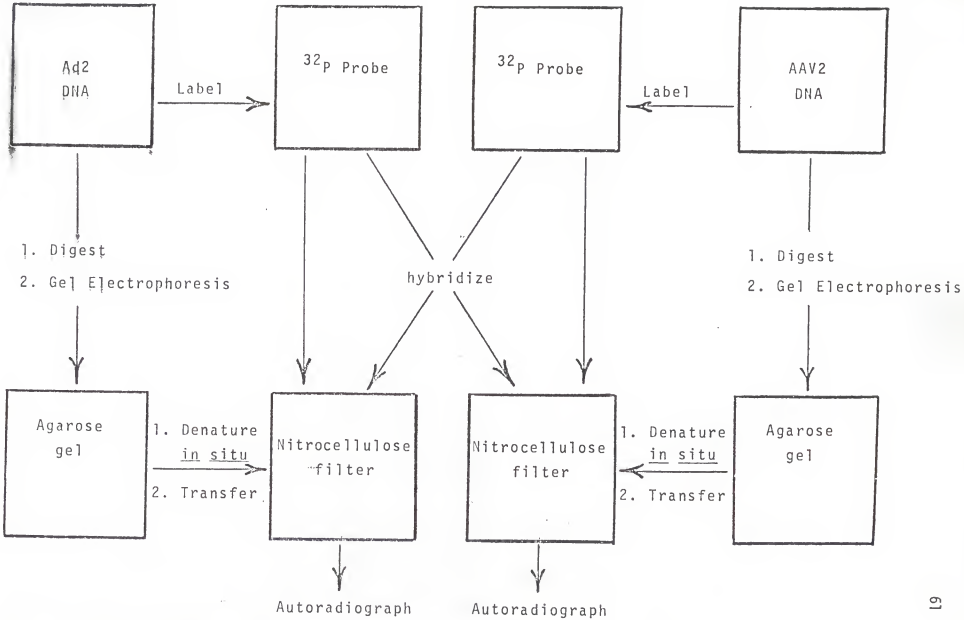

Figure 22. Cross Hybridization Strategy

the experimental group. Figure 23 shows the findings of 2 sets of experiments in which an Ad2 nick translated probe was hybridized to AAV blots and washed under varying salt concentrations. The hybridizations were done overnight (as described in the Materials and Methods portion of this text) at 68°C and all washes were done at 68°C. On the left of Figure 23 are the autoradiographs from the hybridizations to a HpaII blot to AAV and on the right are those from a HaeII blot of AAV. As one progresses from a 2XSSC wash with 300 mM NaCl to a 0.5XSSC wash with only 75 mM NaCl various bands are seen to drop from the autoradiographs. In the case of the HaeII digest, clearly all bands are present in the autoradiograph of the blot washed in 2XSSC but the blot washed in 1XSSC has lost the HaeII D fragment and the intensity of the HaeII B band is greatly diminished. The 0.5XSSC level of stringency reveals only strong hybridization to the HaeII A and C fragments with significant binding remaining to the HaeII E fragment.

When assays are done in the manner described above whole viral DNA is nick translated as a probe. This approach permits the mapping of homologous regions on the nitrocellulose bound DNA but fails to correlate that portion found in the blot with the portion of probe hybridizing in reciprocal crosses. To obtain this information fragments which failed to show homology when bound to nitrocellulose were extracted from gels after endonuclease digestion and gel electrophoresis. These fragments were nick translated and then used as independent probes in cross hybridization assays. Figure 24 shows the results of such an assay using the EcoRl A and C fragments of Ad2 as probes against a HaeII digestion blot of AAV. On the left is the autoradiograph of total AAV hybridized to an EcoRl digest of Ad2 under stringent conditions (hybridization overnight at 68°C in 5XSSC and final overnight wash at 68°C in 0.5XSSC).

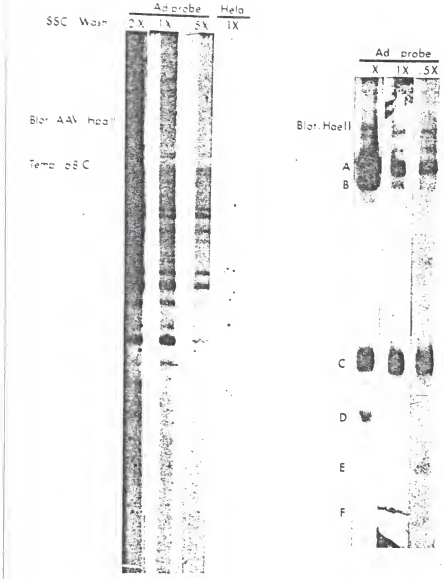

Figure 23. Autoradiograph of Ad2 Probe Hybridized to AAV

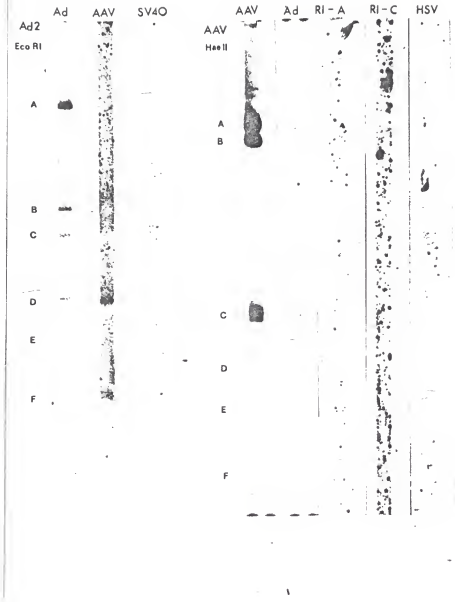

Figure 24. Autoradiograph of AAV/Ad2 Cross Hybridizations

The AAV probe binds only to the EcoRl D and F fragments. A self hybridization with an Ad2 probe was done to confirm that all the digestion fragments were transferred efficiently to the blot. A specificity control is also shown using a nick translated SV40 probe. The EcoRl A and C fragments were extracted from another portion of the gel and nick translated. The findings are shown on the left. A total Ad2 probe bound to the HaeII A and C fragments of AAV. The EcoRl A and C probes of Ad2 showed no homology with AAV under these conditions of hybridization. The HaeII A and C fragments of AAV contain the terminal inverted repeats, which have been shown to have a high G+C content. For this reason HSV-1 DNA, which also has a high G+C content, was used as a control for nonspecific hybridization and none was found.

Because of its defective nature AAV must be grown in the presence of Ad. Unfortunately this situation permits the potential cross contamination of AAV viral stocks with Ad DNA. To limit this occurrence both AAV and Ad DNA stocks were extracted from CsCl gradient purified virions. The possibility still remains that a small portion of the contaminating DNA may have been packaged in the reciprocal viral capsid, thus copurifying throughout the preparative procedure. It may be argued that such an event could in part give rise to the finding demonstrated in the previous experiments. In dealing with this argument two approaches have been taken. First, Ad5 stocks which were maintained continually in the absence of AAV were cut with BamHl giving rise to two fragments of approximately 14.3×10^6 dalton and 9.7×10^6 dalton in size. After electrophoresis the digest was transferred to nitrocellulose and hybridized with a nick translated probe of total AAV which had been grown in coculture with Ad2. Hybridization with the AAV probe involved only the high

molecular weight Ad5 BamHl fragments and showed no smaller contaminating AAV fragments. The second approach was to hybridize Ad DNA from a viral stock shown to be free of AAV by EM studies, to AAV DNA generated from a clone of the entire genome inserted into pBR322. The Ad DNA for this assay was donated by Peter McGuire, Department of Biochemistry, University of Florida, and the 620 pBR322 clone of AAV was a gift from Jude Samulski, Department of Immunology and Medical Microbiology, University of Florida. In both cases, the results confirm the previous findings in the AAV and Ad2 cross hybridizations. The hybridization studies on Ad5 were extended to map the regions of the genome to which binding was most stringent. For this purpose EcoRl and HindIII digests were used and again the salt conditions for the hybridizations or washes varied. The findings are compatible with those previously found for Ad2. A summary of the cross hybridization mapping studies is shown in Figure 25.

To evaluate in better detail the cross hybridization studies a computer comparison was done of the entire nucleotide sequence of AAV-2 against the published sequences of the first 11,600 bases of the left half of Ad2 and the final 8,561 nucleotides of the right half of Ad2. Three programs were run: the first conducted a random search for sequences with a minimum perfect match of six bases, the second searched for a 3 out of 5 base match and the third looked for a 7 out of 10 match. In general the data showed extensive random homology throughout both genomes. This is supportive of the less stringent hybridization studies. If one analyzed the data for sets of adjacent homologies that mapped co-linearly between the two genomes these sequences were invariably found within the terminal 20% of the AAV genome, again corroborating the hybridization data. The following are comparisons of some of the

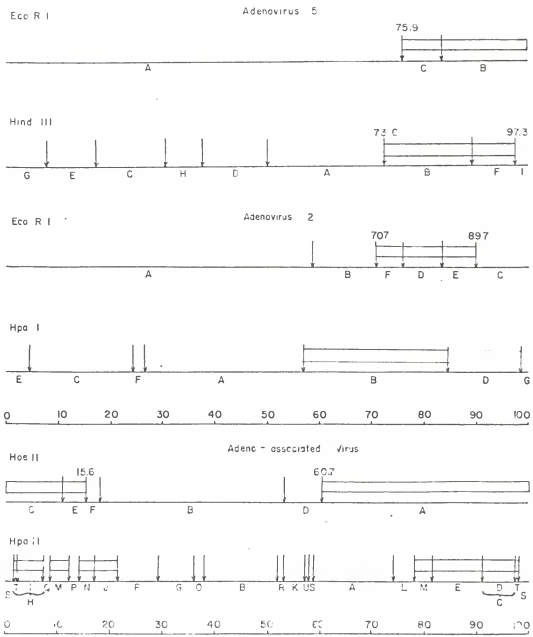

Figure 25. Mapping of Homologous Regions in AAV and Ad

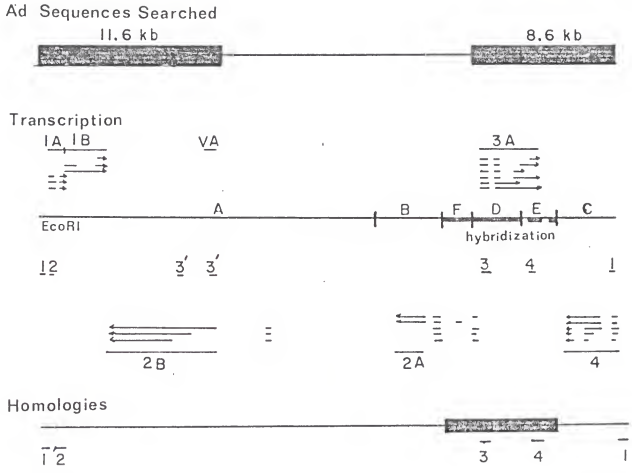

Figure 26. Summary of Ad/AAV Homology

sequences found in this manner. These regions of homology have been numbered and are summarized in Figure 26.

Upon comparison the portion of the Ad2 genome mapping 7930 to 7999 base pairs in from its right terminus shows a 71% sequence homology with the 60 base pair sequence beginning 40 nucleotidesin on the inverted terminal repeat of AAV (see Figure 27). This maps within the EcoR1 D fragment of Ad2 78% of the B and C' domains of AAV are conserved as are the functions between B and B' domains and the C' to A' junction. Eighty-eight percent of the C reiteration of AAV is retained. Approximately 20 nucleotides upstream from the homologous stretch shown the sequence 5' TGCGC 3' is conserved in both genomes; similarly 25 nucleotides downstream in both genomes the sequence 5' CTCCA 3' is retained. The sequence 5' CTCACCGG 3' shown in figure 27 in Ad2 complements the 3' end of the first leader sequence in the E3 transcription complex (Hérissé et al., 1980).

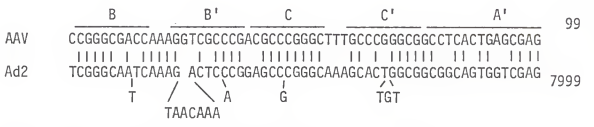

Figure 27. Ad2 Early Region 3A (Hérissé et al., 1980)

Figure 28 details the sequence homology surrounding the beginning of the second coding region in AAV and a homologous stretch in Ad2 starting 5252 nucleotides in from the right terminus. This 53 base pair sequence maps to the EcoR1 E fragment of Ad2 and is part of sequence involved in the E3 transcription complex. There is about 69% total sequence homology between the two genomes over this area and it should be noted that the ATG start signal found in AAV is absent in the

Ad2 sequence. Figure 29 compares a 26 nucleotide sequence found just outside the inverted terminal repeat of AAV starting at base pair 144 to a similar region in the terminal repeat of Ad2. There is an 87% homology between the two regions.

```
AAV    TCAGCCAGGTACATGGAGCTGGTCGGGTGGGTCGTGGACAAGGGGATTACCTCGGAG 1306
         || |||| |   ||| |||||  |||| | | || |   ||| | || ||  ||||
Ad2    GCA CCAG TTTTTGGCGCTGGGTGGGTAGCTTGTAGCTGAGGCGGTTGCC  GGAG 5305
                                                            ↑
                                                            T
```

Figure 28. AAV Coding Region 3 (Hérissé et al., 1980)

```
AAV    CTGGAGGGGTGGAGTCGTGACGTGA 168
         ||  ||||||||||  ||||||||
Ad2    ATGAGGGGGTGGAGTTGTGACGTGG  70
                       ↑
                       T
```

Figure 29. Ad2 Terminal Repetition (Hérissé et al., 1980)

Although cross hybridization assays failed to reveal any homology between HSV and AAV three lines of evidence suggest at least a general similarity in the architecture of their genomes. The termini of both genomes contain a tandem array of natural and inverted repeats. Portions of these repeat regions may be flipped or inverted in their relation to unique regions of the genomes and this flip-flopping appears to be a natural consequence of viral DNA replication. And lastly there is an inherent ability within the replication cycle for maintaining identity between the termini through some mechanism of gene conversion. The reiterated sequences in both viruses probably contain the origin of replication and at least some early regulatory function. A comparison was made between the terminal reiterated sequences of AAV and the published sequence for the L/S junction of HSV-1, which contains the HSV

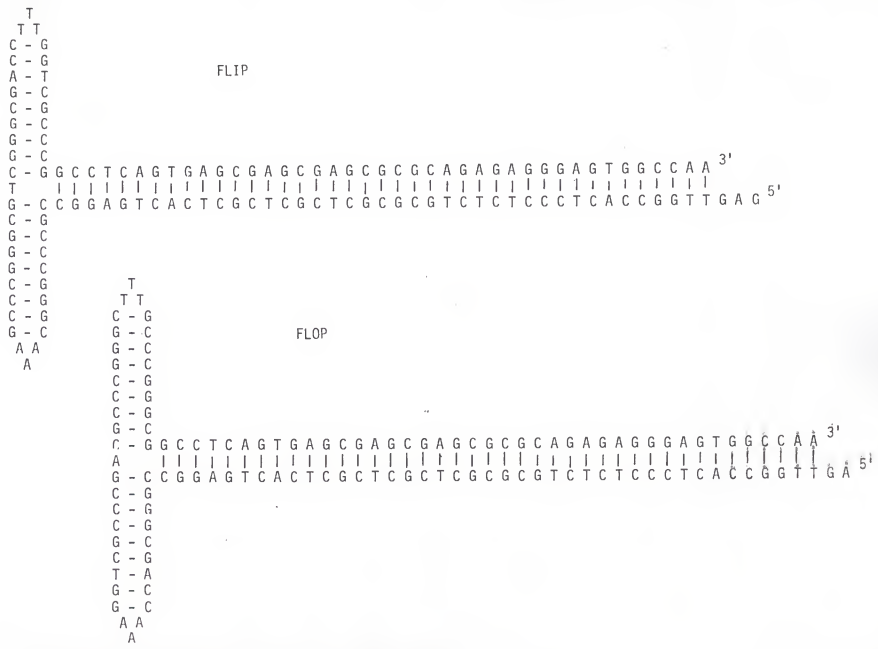

Figure 30. Inversion of portions of the AAV genome

terminal repeats also. Figure 30 presents the "flip" and "flop" orientations of the AAV genome to which the HSV sequences were compared.

The direct repeat which brackets the ends of the a reiteration in the termini of HSV-1 has a similar sequence to the B', C, and C' domains of the AAV hairpin. The short palindrome GCCCGGGC forms the core of this repeat in HSV-1 DNA. This palindrome is found in the core of the inverted repeat of AAV also. One finds that 80% of the base sequence is conserved between the HSV-1 direct repeat and the B', C and C' domains of AAV DNA. The nucleotide sequences bordering the palindrome conserve 66% of the B' and C' repeated domains (see Figure 31).

```
                B          C          C'
AAV     GGTCGCCCGACGCCCGGGCTTTGCCCGGGCG  86
        || |||  |  |||||||||  || | | ||
HSV     GGCCGCGGGGGGCCCGGGCT  GCGCCGCCG  213
                   DR1
```

Figure 31. Homology between short palindromes in HSV-1 and AAV (Mocarski et al., 1980)

By expanding the comparison of this region in HSV with AAV one finds approximately 72% of the flip orientation of the AAV terminal palindrome is conserved in the HSV sequences (see Figure 32). Eighty-two percent of the nucleotides reading from roughly the middle of the A domain of AAV through the C and into the C' domains are the same. Approximately 110 base pairs upstream in the HSV-1 map from the sequence described above one finds a stretch of 43 nucleotides which can be aligned with the flop orientation of the AAV termini. Having done this there appears to be roughly 84% homology between the two genomes in this region. As one continues to read in a 5' to 3' direction the HSV sequence is highly compatible with the terminal domains of AAV arranged in the order A, C, C', B, B', C, C' then A. There is an 84% homology between genomes in

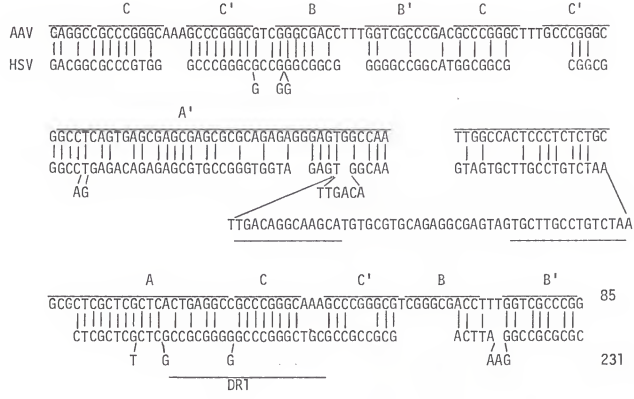

Figure 32. Homology between the terminus of the long unique region in HSV-1 and the terminus of AAV

this first half of this array. The second half of the array is conserved in 68 to 70% of the sequences.

The ends of the HSV sequences displayed in Figure 32 are separated by 20 nucleotides. This boundary is formed in part by a twelve base pair inverted repeat.

As one reads downstream from the end of the preceeding sequence through the A reiteration of HSV, one comes to the direct repeat defining its far terminus. Again this region can be aligned with the terminal AAV sequences. About 63% of the sequence of the A domain is conserved and the short palindrome GCCCGGGC is retained in full within both genomes (see Figure 33). The sequence ACTCC is found 51 base pairs downstream from this short palindrome in both AAV and HSV, followed by the sequence GGTTCCT. In HSV these two short sequences are contiguous while in AAV they are separated by 10 base pairs.

```
                                  A                    C
AAV   CACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCCGCCCGGGCAAAG  54
      | | || | | ||||||| ||| | | | |   ||  ||||||||
HSV   CCCGCCTTTTTTGCGCGC CGC CCCGCCCGCGGGGGGCCCGGGCTGCC 685
                                        DR1
```

Figure 33. Homology between the terminus of the short unique region in HSV-1 and the terminus of AAV (Mocarski et al., 1980)

DISCUSSION

The trigeminal ganglia of 22 patients have been assayed for HSV sequences by DNA/DNA hybridization: None of the patients manifested clinical evidence of recurrence at the time of death nor was a history of recurrence noted. Fourteen of the patients were sero-positive for HSV. A positive serology was assessed as a complement fixation titer of 1:8 or greater. The RNA from the trigeminal ganglia of 16 patients was also surveyed for HSV transcripts by RNA/DNA hybridization.

Hybridizations were conducted in 50% formamide and 5XSSC solution at 37°C. Given a T_m of 97°C in 1XSSC and a G + C value of 68% the calculated value of the T_m for HSV in 5XSSC would be approximately 104°C (Sheldrick and Berthelot, 1974). Allowing for a 0.7°C decrease in the T_m value for each 1% of formamide present and a 1°C lowering for each 1.5% mismatching of base pairs (McConaughy et al., 1969), the initial conditions of hybridization would permit a 21% mismatch in base pairing between the probe and nitrocellulose bound DNA. Subsequent washes in 1XSSC and 50% formamide at 37°C would permit no more than a 16% mismatch to be retained. In like manner it can be calculated that washes in 0.1XSSC solution alone at 68°C would permit no greater than 10% mismatch in base pairing. Consequently, the signal retained in hybridizations between the HSV-1 probe and ganglia containing HSV-2 DNA or transcripts would be greatly subdued. In such instances the strongest binding would occue in the regions of greatest homology between the two types, namely, the termini and L/S junction.

Under any of the above conditions control experiments demonstrated that the HSV-1 nick translated probe failed to bind to DNA from uninfected human ganglia or connective tissue as well as to the DNAs of AAV, SV40 and Ad2. All three of these viruses have a demonstrated potential for latency in human cell lines in vitro. In reconstruction experiments using blots of 6% acrylamide gels, we were able to detect 5 pg of viral DNA in the presence of 10 µg of carrier DNA, a dilution factor of $2x10^6$. Similarly 10 pg of viral DNA could be detected in the presence of 10 µg of carrier when hybridization was to Southern blots of 0.5% agarose gels. Any real differences in the sensitivities of these two approaches is largely indicative of the increased diffusion witnessed when transfer was from low percentage agarose gels. If the DNA content of the normal diploid human cell is 5 pg (Luria and Darnell, 1978), the 10 µg of ganglia DNA normally assayed would represent the product of about $2x10^6$ cells. Thus, given the molecular weight of the HSV genome is 10^8 daltons, the sensitivity of our hybridization assay is such that we can detect roughly 1 copy of the HSV genome per 200 cells.

In 18 of the 22 assays in which trigeminal ganglia were evaluated for the presence of HSV, total ganglionic DNA was cleaved by endonuclease digestion, transferred to nitrocellulose and probed with nick translated HSV DNA. Eleven of these patients were sero-positive for HSV. Nine of the sero-positive were shown to carry HSV DNA and two of these were also shown to be positive for RNA transcripts. The nucleic acid from two of the sero-positive patients failed to demonstrate HSV specific sequences in either DNA or RNA. Only one of the seven sero-negative patients gave evidence of the presence of HSV DNA or RNA; the remainder were negative. Thus, there is a positive correlation between the presence of compliment

fixing antibodies and the presence of HSV specific sequences as measured by this approach.

Two patients warrant some further discussion. Patient #941 was a 1 year old male who died from congenital lobar emphysema. Although both viral DNA and RNA were detected repeatedly in the trigeminal ganglia no humoral immune response to HSV could be determined by either complement fixation or indirect immunofluoresence assays. The patient had not received sufficient immunosuppressive therapy to negate a humoral response nor was there evidence of congenital failure of the humoral immune system. It is possible the primary viral infection of the infant occurred before the humoral immune system was competent enough to mount a response and no subsequent recurrences occurred. No viral DNA was detected in the ganglia from patient #949 although RNA transcripts from the short repeat regions of the HSV genome were demonstrated. It may be that only this portion of the genome was latent in the ganglia; these findings would then mirror the varied sensitivities of the two experimental approaches.

The detection of only part of the HSV genome in several of the ganglia raises the question of whether the remainder of the genome is absent. Brown et al (1979) has reported the detection of latent defective HSV by complementation of the endogenous genome iwth ts mutants of HSV-1 in 8 out of 14 individuals previously negative for spontaneous release of virus. The altered migration or absence of the terminal EcoRI K fragments revealed in patients #228 and #227 could be examples of such an event. The frequency with which large deletions could occur would be greatest in the terminal regions of the linear genome. Conversely, it may be argued that these fragments have undergone an apparent increase in molecular weight due to covalent linkage with host DNA or some other

form of rearrangement. The approach taken here would not resolve this issue. Frequently the regions which we failed to detect gave rise to smaller endonuclease digestion fragments whose copy number would fall below our level of detection. The sensitivity of our assay would allow us to detect a fragment representing 1% of the genome only if it was present in at least 1 copy per cell. The findings in patients #1082 and #1089 probably fall within this category.

In a recent repetition of this approach using human brain tissue Fraser et al (1981) reported the detection of HSV DNA sequences in six of eleven individuals assayed. Hybridization against total viral DNA revealed all or portions of the HSV genome were present in the six positive individuals. Hybridization against the clone pRB115 showed a marked alteration of the electrophoretic migration pattern of the terminal fragment in oen individual and no change in the two other patients.

The findings of the CsCl enrichment studies lead us to consider the possibility that the bands we detected by blot hybridizations are the product of a lytic infection that ensued at the time of death. The positive controls using DNA from a known HSV infection insures that the mechanics of this approach were efficient enough to retain sufficient levels of viral DNA if this were the case. Given the sensitivity of the hybridization assay itself and the negative findings in known sero-positive patients a reasonable argument can be made against an ongoing lytic infection within the ganglia.

RNA from the trigeminal ganglia of 16 patients was screened for HSV specific transcripts by DNA/RNA hybridization. In 8 of these patients ^{125}I-labeled ganglia cytoplasmic RNA was hybridized to either EcoRl or HpaI endonuclease digests of HSV-1 F strain DNA bound to nitrocellulose paper. We were able to demonstrate HSV specific RNA transcripts in two

of three sero-positive patients assayed and one of the five sero-negative patients. The medical history of the single sero-negative patient, #941, has been discussed along with our findings from DNA/DNA hybridizations. From the mapping of those RNA transcripts observed it is clear that the regions of the genome most frequently encountered to date are those containing sequences in the immediate early and early mRNA species of HSV-1 (Clements et al., 1979; Roizman, 1979).

Because of the frequency with which transcripts mapping to the region of the L/S junction were encountered, the cytoplasmic RNA from 9 individuals was used as a probe in hybridizations against endonuclease digests of the clone pRB115. Eight of these patients were sero-positive. None of the patients contained RNA which would hybridize with pRB115. This result was curious in view of the fact that RNA was present which would hybridize to the larger EcoR1 K fragment present in EcoR1 digests of the total HSV genome. It can be argued that the two probes allowed the recognition of a subset of transcripts associated with latency, namely, a set of transcripts mapping to a region of the EcoR1 K fragment not common to the BamH1 P fragment. The region running downstream from the right end of BamH1 P to the right terminus of EcoR1 K is approxiamtely 1.8 kb in size (see Figure 13).

Using the tsB2 mutant Holland et al (1979) has reported finding no mRNA species of greater than 3 kb in this region. This temperature sensitive mutant is one of a group of ts mutants mapping within the sequences of the short reiterated region which code for ICP4 (Schaffer et al., 1973). The viral gene function coding for transition from immediate early to early polypeptide synthesis also maps to this region and is thought to be a function of ICP4 (Preston et al., 1978). Studies of protein synthesis

during temperature shift experiments on this group of mutants suggest that ICP4 is autoregulated (Schaffer et al., 1973). The 5' terminus of IEmRNA-3 which is believed to code for ICP4 lies within the 1.8 kb region described above (Watson and Van de Woude, 1982; Clements et al., 1979; Anderson et al., 1980). Transcription is normally in a leftward direction proceeding into the region of the BamHl P fragment at the joint (Maxam and Gilbert, 1980).

Finally, two ts mutants of ICP4, tsD and tsK, have been assayed for their ability to establish latency in mice; tsK fails to establish latency in mice while tsD will (Stevens and Cook, 1971). Both mutants block viral DNA synthesis. Thus two ts mutants within a single gene within this region can distinguish between viral functions permitting subsequent DNA replication and the establishment of latency. In light of the above findings further evaluation of transcription through this region during latency is warranted, especially in animal models.

Another portion of the genome highlighted by the transcription studies is the region mapping from about 0.08 to 0.65, especially the area from 0.1 to 0.3 (see Figure 13). The bulk of the early viral proteins genes are within these domains (Schaffer et al., 1973). The genes for the viral thymidine kinase and the viral DNA polymerase of both HSV-1 and HSV-2 map within the region 0.27-0.43 (Clements et al., 1977; Schaffer et al., 1973). Spear and Roizman (1972) have reported a correlation between viral induced oncogenic transformation and ICP8 within the region from 0.30 - 0.45 map units. Stevens and Cook (1971) have demonstrated a latency negative phenotype for the mutants tsA, tsS, tsT, and tsI; all of which map within the region 0.1 to 0.6. It is noteworthy that in subsequent mappings of RNA transcripts by in situ hybridization, Galloway and McDougall (1981) have consistantly found the region 0.1 to

0.3 represented in ganglia; other regions including the L/S junction were variably demonstrated. In similar studies of HSV RNA in cervical carcinoma cells sequences spanning map positions 0.07 to 0.4, 0.58 to 0.63, 0.82 to 0.85 and 0.94 to 0.96 were represented (McDougall et al., 1982). These findings are noteworthy because of the collinearity of the HSV-1 and HSV-2 transcription maps. Taken in total, the studies are supportive of our findings and suggest that a subset of the immediate early and early genes are involved in the establishment and maintenance of latency.

The binding of ^{32}P-labeled HSV DNA to restriction endonuclease digests of ganglia DNA, or conversely ^{125}I-labeled ganglia RNA to HSV DNA has permitted several questions to be considered. Those regions of the viral genome which are represented have been determined by simply noting the presence or absence of given viral DNA bands on the autoradiograph. Although it is reasonable to expect the entire genome must be present as a single unit to give rise to recurrence, some cells within the population may carry only portions of the viral genome. It is therefore not surprising to find an apparent unequal molar representation of viral fragments. This is demonstrated by our findings and suggested by previous work on viral thymidine kinase in ganglia (Yamamoto et al., 1977).

Finally, the question of whether latent viral DNA is covalently intefrated into cellular chromosomes was approached. Any digestion fragments which are covalently linked to cellular DNA will migrate in the agarose gels with an apparent increase in molecular weight as a result of the added cellular component. Digests of viral DNA present as a free circular episome would also have an altered migration for the terminal fragments. DNA sequestered in immature nucleocapsids would give rise to banding patterns similar to that of wild type viral DNA. In interpreting such data it should be noted that most virus isolates from ganglia

explants, to date, have yielded unique endonuclease restriction patterns. Thus for alteration in electrophoretic migration to be reasonably component, the shift should be significant and repeatable when the ganglia DNA is restricted with an enzyme such as XbaI which does not reveal strain differences in HSV. When the latent virus is HSV-2 the fidelity of hybridization with the HSV-1 probe could also be significantly reduced. Thus although our data is compatible with integration of HSV into cellular genomes, it is not sufficient to prove it.

Hybridization of ^{125}I-labeled cytoplasmic RNA to digests of HSV DNA demonstrates the presence of viral RNA transcripts in the ganglia. The design of our experiments using several different restriction digests of HSV enabled us to corroborate our findings and more finely map these sequence homologies. Autoradiographic density of hybridized bands permits estimates of the frequency of given RNA species in our sample. For this reason control reactions were run with the ^{125}I-labeled cytoplasmic RNA from both HSV lyticly infected Hep2 cells and uninfected tissue cultures for comparison. This experimental approach answers the question of which regions of the latent HSV genome are undergoing active transcription. The fact that only a subset of the RNA transcripts were found in latently infected ganglia suggests that some HSV genes may be necessary for maintenance of the latent state.

A number of general observations have prompted the inclusion of a comparative analysis of the HSV, Ad, and AAV genomes within this study of HSV latency. The genomes studies are representative of the three linear DNA virus families whose members are known to replicate within the nuceli of human cells. Architecturally each of these linear genomes contains inverted repeats within their terminal regions. Each of the viruses have a demonstrated potential in vitro for latency in human cells.

And lastly, the defective parvovirus (AAV) is absolutely dependent upon coinfection with either an edenovirus or herpesvirus for its multiplication.

In the absence of a coinfecting helper, AAV enters the cell where its virion is uncoated within the nucleus. No detectable macromolecular synthesis occurs at this time. The naked AAV DNA may then establish a stable relationship with the host cell (Hoggan et al., 1966; Cheung et al., 1980). Subsequent infection with a helper virus promotes the multiplication of infectious AAV within these latent cells. Although the nature of this stable relationship between AAV and the host cell is complex, it is clear that at least a portion of the genome is covalently linked to cellular DNA and in part copies of the entire genome are tandeomly arrayed within this linkage with cellular DNA (Handa and Shimogo, 1977; Cheung et al., 1980). Our findings demonstrate a substantial sequence homology within apparent regulatory regions between the defective genome and those of its helpers. This is of particular interest since no biochemical relatedness has been previously demonstrated between these viruses and the principal role the helper plays in this partnership is the promoting of AAV macromolecular synthesis.

Although 6 to 12 base pair stretches of homology with AAV were found randomly throughout the Ad genome these homologies were concentrated largely within the terminal sequences of the AAV genome. The strongest co-linear homologies between regions of the two genomes which at present appear to have no functional relatedness, involve the EcoRl D and F fragments of Ad and the terminal repitition of AAV. Two examples of sequence homology within these regions have been described. The first of these analyzes the area surrounding the 3' end of the first leader sequence in the E3 transcription complex of Ad and the termini

of AAV. The second compares the beginning of the second coding region in AAV and a portion of the E3 transcription complex. The second example actually lies within the potential coding sequence of the 14K E3 polypeptide of Ad (Hérissé et al., 1980). Because the function of E3 region of Ad is obscure, it is difficult to evaluate these homologies. There is no evidence to date which directly ascribes a specific regulatory role during transcription to the terminal repetition in AAV nor has a role been assigned to the E3 transcription products of Ad. Transcription of the E3 complex is nonessential for growth of Ad or AAV in vitro (Richardson and Westphal, 1981).

Two other short stretches of homology hying within the left half of the Ad genome were also found. The first of these is the 26 bp stretch in AAV (142-168) which is complimentary to a portion of the inverted terminal repeat of Ad. The second is the AAV sequence surrounding the TATA box 31 bases upstream from the leftward most site of initiation of transcription. It has a 17/19 base homology with a comparable sequence upstream from the initiation of the Ad5 early region 1A transcripts. Lusby et al (1980) had previously reported these are example regions with a potential role in transcriptional regulation. Taken in total the data suggest a conservation through evolution of regions of these genomes involved in transcriptional regulation.

As noted previously several lines of evidence suggest at least a general similarity in the architecture of the AAV and HSV genomes. The termini of both genomes contain a tandem array of natural and inverted repeats. Also portions of these repeated regions may be inverted in their relationship to unique regions of the genome as a natural consequence of viral DNA replication (Lusby et al., 1980; Mocarski et al.,

1980). And there is apparently an inherent ability within the replicative cycle for maintaining identity between the termini through some mechanism of gene conversion. Probable origins of replication and at least some early regulatory functions lie within the reiterated sequences in both viruses.

The nucleotide sequence of the natural terminal repeat in HSV-1 F was used for the comparison with the terminal 200 nucleotides of AAV described in this study. The terminal 200 nucleotides of AAV contain the 143 bp inverted terminal repeat. This region can further be broken into two smaller internal palindromic sequences which are bracketed by a larger repeat and its inverse (Lusby et al., 1980). The 5001 bp natural repeat in HSV-1 F is inverted to form the junction of the long and short unique regions of the genome; thus each unique arm of the genome is in turn roughly similar in architecture to the AAV genome, as is the whole. The 501 bp sequence is composed of 20 direct repeats of a 12 nucleotide sequence, 3 repeats of a 37 base sequence and some internally unique sequences; the entire domain is bracketed by a 20 base pair direct repeat (Mocarski et al., 1980).

Our findings demonstrate an extensive homology between portions of the HSV inverted repeats found at the L/S junction and termini with the termini of AAV. The regions of HSV homology are 1) those regions of the "b" reiteration which bracket the long unique region and are immediately adjacent to the "a" repeat, 2) those portions of the "c" reiteration which bracket the short unique region and are contiguous with the "a" repeat, and 3) the 20 base direct repeat (DR1) which brackets the "a" repeat. The high G + C content and repetiveness of these regions would explain our earlier failure to find these homologies by Southern hybridization.

The extensive homology between DRI and the small internal palindromes of the AAV sequence is of particular interest. The short palindrome GCCCGGGC has been totally conserved within both. A role for DRI has been proposed in both the site specific cleavage of concatomeric replicative intermediates of HSV and the inversion of the long and short domains (Mocarski et al., 1980). Similary, deletion mutants within this sequence in AAV are unable to resolve high molecular weight intermediates in DNA replication. Fifty-one base pairs downstream from GCCCGGGC in AAV one finds the sequence ACTCC followed nine base pairs further downstream by the sequence GGTTCCT; ACTCC is the probable cleavage site for the inversion of the AAV terminal palindrome and resolution of the hairpin during DNA replication. The sequence ACTCC followed by GGTTCCT is also found within the "c" reiteration of HSV-1 F fifty-one bases downstream from the sequence GCCCGGGC within DRI. The question of whether this arrangement is coincidental or the sequence actually serves a similar function in the replication of both genomes will require genetic analysis. It should be noted that HSV-1 F strain DNA contains a single copy of the "a" reiteration at its right terminus and the putative cleavage site for resolution of concatomers is thought to lie within or just outside the "a" reiteration adjacent to the "c" repeat (Mocarski et al., 1980).

The terminal sequences of AAV DNA appear to be intimately involved in the integration of latent AAV DNA into cellular chromatin. Endonuclease analysis of AAV DNA from early (10) and late (118) passages of latently infected cells demonstrated that the only sequences altered during clonal passage were those within the palindromic region of the terminal repeat, specifically, the sequence GCCCGGGCT which contains the

SmaI recognition site (Cheung et al., 1980). Thus these sequences could potentially serve a similar role in latency of HSV.

The conservation of similar GC content and architecture between the termini of several parvoviruses supports the genetic evidence assigning identical biological roles to these regions. These observations appear to signify the direct involvement of the termini in viral multiplication. Further the potential to form nearly identical curciform structures through secondary folding of their 3' termini appears to be stringently conserved even within those genomes which have significant sequence divergence from that of AAV. Thus the evolution of this family of small DNA viruses has conserved both primary sequence and secondary structure within a region of the genome in which both must interact in the preservation of fidelity in viral replication.

The question of whether sequence homology between AAV and HSV is a consequence of similar function warrants further investigation in light of the findings within the parvovirus family. Our observations to date do not permit the assumption that homologous sequences serve identical functions within AAV and HSV. A cursory analysis of the homologous sequences in HSV reveals that they are not self complimentary and cannot easily be ascribed a secondary structure similar to AAV. Determination of whether the primary sequence within HSV would lend itself to the formation of a stable curciform secondary structure is central to this line of investigation. In conclusion the sequence homology between AAV and HSV demonstrates the potential for similar mechanics of early regulation and macromolecular synthesis, events which appear to be concomitant with the establishment and maintenance of latency.

LIST OF REFERENCES

Anderson, K., J. Stringer, L. Holland and E. Wagner. 19791 Isolation and localization of herpes simplex virus type I mRNA. Journal of Virology. 30: 805-820.

Anderson, K., L. Holland, B. Gaylord and E. Wagner. 1980. Isolation and transcription of mRNA encoded by a specific region of the Herpes Simplex virus type I genome. Journal of Virology. 33: 749-759.

Atkinson, M.A., S. Barr and M.C. Timbury. 1978. The fine structure of cells infected with termperature sensitive mutants of herpes simplex virus type 2. Journal of General Virology. 2: 517-536.

Bachenheimer, S. and B. Roizman. 1972. Ribonucleic acid synthesis in cells infected with herpes simplex virus. Journal of Virology. 10: 875-879.

Baringer, J.R. 1974. Recovery of herpes simplex virus from human sacral ganglions. New England Journal of Medicine. 291: 828-830.

Baringer, J.R. 1975. Herpes simplex virus infection of nervous tissue in animals and man. Progress in Medical Virology. 20: 1-26.

Baringer, J.R. and P. Sworeland. 1973. Recovery of herpes simplex virus from human trigeminal ganglions. New England Journal of Medicine. 288: 546-650.

Bastian, R.O., A.S. Rabson, C.L. Yee and T.S. Tralka. 1972. Herpes hominis isolation from human trigeminal ganglion. Science. 178: 305-307.

Becker, Y., H. Dym and I. Sarov. 1968. Herpes Simplex virus DNA. Virology. 36: 184-192.

Berns, K.I. and W. Hauswirth. 1978. Parvovirus DNA structure and replication. Replication of Mammalian Parvoviruses. Cold Spring Harbor Laboratory Press. pp. 13-32.

Biswal, N., B. Murray and M. Benyesh-Melnick. 1974. Ribonucleotides in newly synthesized DNA of herpes simplex virus. Virology. 61: 87-99.

Brown, S.M., J.H. Subak-Sharpe, K.G. Warren, Z. Wroblewska, and H. Koprowski. 1979. Detection of uninducible herpes simplex virus genomes latnet in human ganglia explants. Proceedings of the National Academy of Science, U.S.A. 76: 2364-2368.

Buchman, T.G., B. Roizman, G. Adams and B.H. Storer. 1978. Restriction endonuclease fingerprinting of herpes simplex virus DNA: a novel epidemiological tool applied to a nosocomial outbreak. Journal of Infectious Diseases. 138: 488-498.

Bünemann, H. and W. Müller. 1978. Base specific fractionation of double stranded DNA: affinity chromatography on a novel type of absorbant. Nucleic Acids Research. 5: 1059-1074.

Carton, C.A. and E.D. Kilborne. 1952. Activation of latent herpes simplex by trigeminal sensory-root section. New England Journal of Medicine. 246: 172-176.

Centifanto-Fitzgerald, Y., E. Varnell and H. Kaufman. 1982. Initial herpes simplex virus type 1 infection prevents ganglionic superinfection by other strains. Infection and Immunity. 35: 1125-1132.

Centifanto-Fitzgerald, Y., T. Yamaguchi, H. Kaufman, M. Tognon and B. Roizman. 1982. Ocular disease pattern induced by herpes simplex virus is genetically determined by a specific region of viral DNA. Journal of Experimental Medicine. 155: 475-489.

Cheung, A., D. Hoggan, W. Hauswirth and K.I. Berns. 1980. Integration of the adeno-associated virus genome into cellular DNA in latently infected human Detroit 6 cells. Journal of Virology. 33: 730-748.

Clements, J., R. Watson, and N. Wilkie. 1977. Temporal regulation of herpes simplex virus type I transcription: location of transcripts on the viral genome. Cell. 12: 275-285.

Clements, J.D., T. McLaughlan and D.S. McGeoch. 1979. Orientation of herpes simplex virus type 1 immediate early mRNAs. Nuclear Acid Research. 7: 77-93.

Comerford, S. 1971. Iodination of Nucleic Acids in vitro. Biochemistry. 10: 1993-1995.

Cook, M.L., V.B. Bastone and J.G. Stevens. 1974. Evidence that neurons harbor latent herpes simplex virus. Infection and Immunology. 9: 946-951.

Cook, M.L. and J.G. Stevens. 1973. Pathogenesis of herpetic neuritis and ganglionitis in mice: evidence for intra-axonal transport of infection. Infection and Immunity. 7: 272-288.

Cook, M.L. and J.G. Stevens. 1976. Latent Herpetic infections following experimental viremia. Journal of General Virology. 31: 75-80.

Daniels, C.A., S.G. LeGoff, and A.L. Notkins. 1975. Shedding of infectious virus-antibody complexes from vesticular lesions of patients with recurrent herpes labialis. Lancet. 2: 524-528.

Delius, H. and J. Clements. 1976. A parital denaturation map of herpes simplex virus type 1 DNA: evidence for inversion of the unique DNA regions. Journal of General Virology. 33: 125-133.

Ecob-Johnston, M.S. and W.O. Whetsell. 1979. Host-cell response to herpes virus infection in central and peripheral nervous tissue in vitro. Journal of General Virology. 44: 747-757.

Feldman, L.A., R.D. Sheppard and M.B. Bronstein. 1968. Herpes simplex virus-host cell relationships in organized cultures of mammalian nerve tissues. Journal of Virology. 2: 621-628.

Finkelstein, M. and R. Rownd. 1978. A rapid method for extracting DNA from agarose gels. Plasmid. 1: 557-562.

Fraser, N., W. Lawrence, Z. Wroblewska, D. Gilden, and H. Koprowski. 1981. Herpes Simplex type 1 DNA in human brain tissue. Proceedings National Academy of Science, U.S.A. 78: 6461-6465.

Frenkel, N. and B. Roizman. 1972. Separation of herpes virus DNA duplex into unique fragments and intact strand on sedimentation in alkaline gradients. Journal of Virology. 10: 565-572.

Frenkel, N., H. Locker, W. Batterson, G.S. Hayward and B. Roizman. 1976. Anatomy of herpes simplex virus DNA VI. Defective DNA originates from the S component. Journal of Virology. 20: 527-531.

Galloway, D.A., C. Genoglio, M. Shevchuk and J.K. McDougall. 1979. Detection of herpes simplex RNA in human sensory ganglia. Virology. 95: 265-268.

Galloway, D. and J. McDougall. 1981. Transformation of rodent cells by a cloned DNA fragment of Herpes Simplex type 2. Journal of Virology. 38: 749-760.

Goodpasture, E.W. 1929. Herpetic infection with especial reference to involvement of the nervous system. Medicine. 8: 223-243.

Grafstrom, R.H., J.C. Alwine, W.L. Steinhart and C.S. Hill. 1974. Terminal repetitions in herpes simplex virus type I DNA. Cold Springs Harbor Symposium Quart. Biol. 39: 679-681.

Graham, B., H. Ludwig, D. Bronson, M. Benyesh-Melnick and N. Biswal. 1972. Physiochemical properties of the DNA of herpes viruses. Biochemical and Biophysical Acta. 259: 13-23.

Handa, H. and H. Shimojo. 1977. Isolation of the viral DNA replication complex from adeno-associated virus type 1 infected cells. Journal of Virology. 24: 444-450.

Hayward, G.S., N. Frenkel and B. Roizman. 1975. Anatomy of herpes simplex virus DNA: strain differences and heterogeneity in the location of restriction endonuclease cleavage sites. Proceedings of the National Academy of Science. 72: 1768-1772.

Hayward, G.S., R.J. Jacob, S.C. Wadsworth, and B. Roizman. 1975. Anatomy of herpes simplex virus: Evidence for four populations of molecules that differ in the relative orientations of their long and short components. Biochemistry. 72: 4243-4247.

Hérissé, J., G. Courtois and F. Galibert. 1980. Nucleotide sequence of the EcoRI D fragment of Adenovirus z genome. Nucleic Acid Research. 8: 2173-2191.

Hérissé, J. and F. Galibert. 1981. Nucleotide sequence of the EcoRI E fragment of adenovirus 2 genome. Nucleic Acid Research. 9: 1229-1240.

Hill, T.S. and H.S. Field. 1973. The interaction of herpes simplex virus with cultures of peripheral nervous tissue: an electron microscope study. Journal of General Virology. 21: 123-133.

Hill, T.S., H.S. Field and W.A. Blyth. 1975. Acute recurrent infection with herpes simplex virus in the mouse: a model for studying and recurrent disease. Journal of General Virology. 28: 341-353.

Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. Journal of Molecular Biology. 26: 365-369.

Hoggan, M., N. Blacklow and W. Rowe. 1966. Studies of small DNA viruses found in various adenovirus preparations: Physical, biological and immunological characteristics. Proceedings of the National Academy of Science, U.S.A. 55: 1467-1575.

Holland, L.E., K.P. Anderson, J.R. Stringer and E.K. Wagner. 1979. Isolation and localization of herpes simplex virus type 1 mRNA abundant before viral DNA synthesis. Journal of Virology. 31: 447-462.

Honess, M. and D. Watson. 1974. Herpes simplex virus-specific polypeptides studied by polyacrylamide gel electrophoresis of immune precipitates. Journal of General Virology. 22: 171-183.

Jones, P. and B. Roizman. 1979. Regulation of Herpesvirus macromolecular synthesis VIII. The transcription program consists of three phases during which both extent of transcription and accumulation of RNA in the cytoplasm are regulated. Journal of Virology. 31: 299-314.

Kaplan, A.S. (ed.) 1973. The Herpesviruses Academic Press, New York.

Kieff, E.D., S.L. Bachenheimer and B. Roizman. 1971. Size, composition and structure of the deoxyribonucleic acid of herpes simplex virus subtypes 1 and 2. Journal of Virology. 8: 125-132.

Knipe, D., W. Ruyechan, R. Honess and B. Roizman. 1979. Molecular genetics of herpes simplex virus: The terminal a sequences of the L and S components are obligatorily identical and constitute a part of a structural gene mapping predominantly in the S component. Proceedings of the National Academyof Science, U.S.A. 76: 4534-4538.

Kristensson, K., B. Ghetti and H.M. Wisniewski. 1974. Study on the propagation of herpes simplex virus (type 2) into the brain after intraocular injection. Brain Research. 69: 189-201.

LePecq, J. 1970. Use of ethidium bromide for separation and determination of nucleic acids of various conformational forms and measurement of their associated enzymes. Methods of Biochemical Analysis. 20: 41-86.

Lis, J.T. 1980. Fractionation of DNA fragments by polyethylene glycol induced precipitation. Methods in Enzymology. 65: 347-353.

Locker, H. and N. Frenkel. 1979. BamI, KpnI and SalI restriction enzyme maps of the DNAs of herpes simplex virus strains Justin and F: occurrence of heterogeneities in defined regions of the viral DNA. Journal of Virology. 32: 429-441.

Lonsdale, D.M., S.M. Brown and J.H. Subak-Sharpe. 1979. The polypeptide and the DNA restriction enzyme profiles of spontaneous isolates of herpes simplex virus type 1 from explants of human trigeminal, superior cervical and vagus ganglia. Journal of General Virology. 43: 151-171.

Luria, S. and J. Darnell. 1978. General Virology. 3rd ed. Wiley. New York, New York.

Lusby, E., K. Fife and K. Berns. 1980. Nucleotide sequence of the inverted terminal repetition in adeno-associated virus DNA. Journal of Virology. 34: 402-409.

Lusby,E. and K. Berns. 1982. Mapping the 5' termini of two adeno-associated virus RNAs in the left-half of the genome. Journal of Virology. 41: 518-524.

McConaughy, B.L., C.D. Laird and B.S. McCarthy. 1969. Nucleic acid reassociation in formamide. Biochemistry. 8: 3289-3294.

McDougall, J., C. Crum, C. Fenoglio, L. Goldstein, and D. Galloway. 1982. Herpesvirus-specific RNA and protein in carcinoma of the uterine cervix. Proceedings of National Academy of Science, U.S.A. 79: 3853-3857.

McLauchlan, J. and J.B. Clements. 1982. A 3' co-terminus of two early herpes simplex virus type 1 mRNAs. Nucleic Acid Research. 10: 501-512.

Maxam, A.M. and W. Gilbert. 1980. Sequencing end-labeled DNA with base-specific chemical cleavages. Methods of Enzymology. 65: 499-460.

Mocarski, E.S., L.E. Post and B. Roizman. 1980. Molecular Engineering of the herpes simplex virus genome: insertion of a second L-S junction into the genome causes additional genome inversions. Cell. 22: 240-255.

Morse, L., L. Pereira, B. Roizman, and P. Schaffer. 1977. Anatomy of HSV DNA. VI Mapping of viral genes by analysis of polypeptides and functions specified by HSV-1 X HSV-2 recombinants. Journal of Virology. 26: 389-410.

Moss, B., A. Gershowitz, J. Stringer, L. Holland, and E. Wagner. 1977. 5'-Terminal and internal methylated nucleosides in herpes simplex virus type 1 mRNA. Journal of Virology. 23: 234-239.

Naragi, S., G.G. Jackson and Q.M. Jonasson. 1976. Viremia with herpes simplex type-1 in adults. Annals of Internal Medicine. 85: 165-169.

Nesburn, A., M. Cook and J. Stevens. 1976. Latent herpes simplex virus. Isolation from rabbit trigeminal ganglia between episodes of recurrent ocular infection. Archives of Ophthalmology. 88: 412-418.

Nii, S., C. Morgan and H.M. Rose. 1968. Electron microscopy of herpes simplex virus II: sequence of development. Journal of Virology. 2: 517-536.

Pater, M.M., R.W. Hyman and F. Rapp. 1976. Isolation of herpes simplex virus DNA from the "Hirt Supernatant". Virology. 75: 481-483.

Pazin, G., M. Ho and P. Jannetta. 1978. Reactivation of herpes simplex virus after decompression of the trigeminal nerve root. Journal of Infectious Diseases. 138: 405-409.

Pereira, L., M. Wolff, M. Fenwick and B. Roizman. 1977. Regulation of herpesvirus macromolecular synthesis V. Properties of α polypeptides made in HSV-1 and HSV-2 infected cells. Virology. 77: 733-749.

Pignatti, P.F., E. Cassai, G. Memequzzi, N. Chenciner and G. Milanesi. 1979. Herpes simplex virus DNA isolation from infected cells with a novel procedure. Virology. 93: 260-264.

Plummer, G., J. Wagner, A. Phuargsalo and C. Goodheart. 1970. Type 1 and Type 2 herpes simplex viruses: serological and biological differences. Journal of Virology. 5: 51-59.

Post, L.E., A.J. Conley, E.S. Mocarski and B. Roizman. 1980. Cloning of the reiterated and non-reiterated herpes simplex virus 1 sequences as BamHl fragments. Proceedings of the National Academy of Science, U.S.A. 77: 4201-4205.

Post, L., S. Mackem, and B. Roizman. 1981. Regulation of α genes of herpes simplex virus: expression of chimeric genes produced by fusion of thymidine kinase with γ gene promoters. Cell. 24: 555-565.

Preston, V., A. Davison, H. Marsden, M. Timbury, J. Subak-Sharpe, and N. Wilkie. 1978. Recombinants between Herpes simplex virus types 1 and 2: Analysis of genome structures and expression of immediate early polypeptides. Journal of Virology. 28: 499-571.

Price, R.W. and A.L. Notkins. 1977. Viral infections of the autonomic nervous system and its target organs: pathogenetic mechanisms. Medical Hypothesis. 3: 33-36.

Price, R.W. and J. Schmitz. 1978. Reactivation of latent herpes simplex virus infection of the autonomic nervous system by postganglionic neuroctomy. Infection and Immunity. 19: 523-533.

Puga, A., J.D. Rosenthal, H. Openshaw and A.L. Notkins. 1978. Herpes simplex virus DNA and mRNA sequences in acutely and chronically infected trigeminal ganglia of mice. Virology. 89: 102-111.

Rajcani, F. 1978. Experimental pathogenesis of non-lethal herpes virus infection and the establishment of latency. Acta Virology. 22: 278-286.

Richardson, C.C. 1966. The 5'-terminal nucleotides of T_7 bacteriophage deoxyribonucleic acid. Journal of Molecular Biology. 15: 49-61.

Richardson, W. and H. Westphal. 1981. A cascade of adenovirus early functions is required fro expression of adeno-associated virus. Cell. 27: 131-140.

Rigby, P.W.S., M. Dieckmann, C. Rhodes and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. Journal of Molecular Biology. 113: 237-251.

Rixon, F. and J.B. Clements. 1982. Detailed structural analysis of two spliced HSV-1 immediate-early mRNAs. Nucleic Acids Research. 10: 2241-2256.

Roizman, B. 1979. The structure and isomerization of herpes simplex virus genome. Cell. 16: 481-494.

Roizman, B. and P. Spear. 1968. Preparation of herpes simplex virus of high titer. Journal of Virology. 2: 83-84.

Roizman, B., M. Kozak, W. Honess and G. Hayward. 1974. Regulation of herpesvirus macromolecular synthesis: Evidence for multilevel regulation of herpes simplex 1 RNA and protein synthesis. Cold Spring Harbor Symp. Quant. Biology. 39: 687-702.

Rustigan, R., J.B. Smalow, M. Type, W.A. Gibson and E. Shindell. 1966. Studies on latent infection of skin and oral mucosa in individuals with recurrent herpes simplex. Journal of Investigative Dermatology. 47: 218-221.

Schaffer, P., G. Aron, N. Biswal and M. Benyesh-Melnick. 1973. Temperature-sensitive mutants of herpes simplex virus type 1: Isolation complementation and partial characterization. Virology 52: 57-71.

Scott, T. and T. Tokumaru. 1964. Herpesvirus hominis (virus of herpes simplex). Bacteriological Reviews. 28: 458-471.

Sheldrick, P. and N. Berthelot. 1974. Inverted repetitions in the chromosome of herpes simplex virus. Cold Spring Harbor Symposium Quantitative Biology. 39: 667-678.

Silverstein, S., R. Millette, P. Jones and B. Roizman. 1976. RNA synthesis in cells infected with herpes simplex virus. XII Sequence simplexity and properties of RNA differing in extent of adenylation. Journal of Virology. 18: 977-991.

Skare, J., W.P. Summers and W.C. Summers. 1975. Structure and function of herpes virus genomes I. Comparison of five HSV-1 and two HSV-2 strains of cleavage of their DNA with EcoR1 restriction endonuclease. Journal of Virology. 15: 726-732.

Skare, J. and W. Summers. 1977. Structure and function of herpesvirus genomes II. EcoRI, XbaI and HindIII endonuclease cleavage sites on herpes simplex virus type 1 DNA. Virology. 76: 581-595.

Smith, H. and M.L. Birnstiel. 1976. A simple method of DNA restriction site mapping. Nucleic Acid Research. 3: 2387-2398.

Southern, E.M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. Journal of Molecular Biology. 98: 503-517.

Spear, P. and B. Roizman. 1972. Proteins specified by herpes simplex viruses. V. Purification and structural proteins of the herpes virions. Journal of Virology. 9: 431-439.

Stevens, J.G. 1975. Latent herpes simplex virus and the nervous system. Current Topics in Microbiology and Immunology. 70: 31-50.

Stevens, J.G. and M.L. Cook. 1972. Latent herpes simplex virus in spinal ganglia of mice. Science. 173: 843-845.

Stevens, J.G. and M.L. Cook. 1972. Latent herpes simplex virus from trigeminal ganglia of rabbits with recurrent eye infections. Nature New Biology. 235: 216-217.

Subak-Sharpe, J., S. Brown, D. Ritchie, M. Timbury, J. Macnab, H. Marsden and J. Hay. 1974. Genetic and biochemical studies with herpesviruses. Cold Spring Harbor Symposia on Quantitative Biology. 39: 717-729.

Tereba, A. and B.J. McCarthy. 1973. Hybridization of ^{125}I-labeled ribonucleic acid. Biochemistry. 12: 4675-4679.

Thomas, C.A. and L.A. MacHattie. 1967. The anatomy of viral DNA molecules. Annual Reviews of Biochemisty. 36: 485.

Vlanzy, D., A. Kwong and N. Frenkel. 1982. Site specific cleavage/packaging of herpes simplex virus DNA and the selective maturation of nucleocapsids containing full length viral DNA. Proceedings of the National Academy of Science, U.S.A. 79: 1423-1427.

Wadsworth, S., R.J. Jacob and B. Roizman. 1975. Anatomy of herpes virus DNA. II. Size, composition and arrangement of inverted terminal repetitions. Journal of Virology. 15: 1487-1497.

Wagner, E. and B. Roizman. 1969. RNA synthesis in cells infected with herpes simplex virus II. Evidence that a class of viral mRNA is derived from a high molecular wright precursor synthesized in the nucleus. Proceedings of the National Academy of Science, U.S.A. 64: 626-633.

Wagner, N.M. and W.C. Summers. 1978. Structure of the joint region and the termini of the DNA of herpes simplex virus type I. Journal of Virology. 27: 374-387.

Wahl, G.M., M. Stern and G.R. Stark. 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl-paper and rapid hybridization by using dextran sulfate. Proceedings of the National Academy of Sciences. 78: 3683-3687.

Walboomers, J. and J. Ter Schegget. 1976. A new method for the isolation of herpes simplex virus type 2 DNA. Virology. 74: 256-258.

Walz, M.A., R.W. Price and A.L. Notkins. 1974. Latent ganglionic infection with herpes simplex virus types 1 and 2: viral reactivation in vitro after neurectomy. Science. 184: 1185-1187.

Walz, M.A., H. Yamamoto and A.L. Notkins. 1976. Immunological response restricts number of cells in sensory ganglia infected with herpes simplex virus. Nature. 264: 554-556.

Warren, K.G., S.M. Brown, Z. Wroblewska, D. Gilden, H. Koprowski and J. H. Subak-Sharpe. 1978. Isolation of latent herpes virus from the superior cervical and vagus ganglia of humans. New England Journal of Medicine. 298: 1068-1069.

Watson, R.J. and G.F. VandeWoude. 1982. DNA sequence of an immediate-early gene (IEmRNA-5) of herpes simplex virus type I. Nucleic Acid Research. 10: 979-991.

Wetmur, J. 1975. Acceleration of DNA renaturation rates. Biopolymers. 14: 2517-2524.

Wilkie, N. 1973. The synthesis and sub structure of herpes virus DNA: the distribution of alkali-labile single strand interruptions in HSV-1 DNA. Journal of General Virology. 21: 453-467.

Wilkie, N. 1976. Physical maps for herpes simplex virus DNA for restriction endonucleases HindIII, HpaI, and XbaI. Journal of Virology. 20: 222-233.

Wilkie, N.M., J.B. Clements, J.C.M. MacNab and J.H. Subak-Sharpe. 1974. The structure and biological properties of herpes simplex virus DNA. Cold Spring Harbor Symp. Quant. Biol. 39: 657-666.

Wilkie, N. and R. Cortini. 1976. Sequence arrangement in herpes simplex virus type 1 DNA: identification of terminal fragments in restriction endonuclease digests and evidence for inversions in redundant and unique sequences. Journal of Virology. 20: 211-221.

BIOGRAPHICAL SKETCH

I was born in Miami, Florida, on April 1, 1953, the first child of Amos and Fran Rayfield. Our family was completed in 1956 with the birth of my brother, Glenn. I attended the public school system of Dade County and eventually enrolled at the University of Florida in the Department of Immunology and Medical Microbiology as an undergraduate.

While an undergraduate, I worked in the Department of Ophthalmology in the virology laboratory of Dr. Centifanto. I decided to further my education in this field with a specific interest in HSV. I was accepted into the Department of Immunology and Medical Microbiology and over the years worked with various viruses which led to this dissertation.

I recently married Dorothy Lesso, a student at the University of Florida, College of Environmental Engineering. Our plans include a move to New Orleans where I am currently a post doctorate fellow at the LSU Eye Institute.

Basically I'm an outdoors man and my interests include a spectrum of sports which my wife and I share.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Kenneth I. Berns, M.D., Ph.D., Chairman
Professor and Chairman of Immunology
and Medical Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Kenneth H. Rand, M.D., Ph.D.
Associate Professor of Immunology and
Medical Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William W. Hauswirth, Ph.D.
Associate Professor of Immunology and
Medical Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Nicholas Muzyczka, Ph.D.
Assistant Professor of Immunology and Medical Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

William K. Holloman, Ph.D.
Assistant Professor of Immunology and Medical Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Gary S. Stein, Ph.D.
Professor of Biochemistry and Molecular Biology

This dissertation was submitted to the Graduate Faculty of the College of Medicine and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December, 1982

Dean, College of Medicine

Dean, Graduate School